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INVESTIGATIONS CONCERNING HYDROLYSIS AND STABILIZATION OF ANTIRADIATION COMPOUNDS

ANNUAL REPORT

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January 1983

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CHEMISTRY AND CHEMICAL ENGINEERING DIVISION

August 16, 1985

Director
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Subject: Annual Report - Contract DAMD17-80-C-0128
SwRI Project 01-6223
Investigation Concerning the Hydrolysis and Stabilization aof Antiradiation Compounds

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Attached as you requested are 12 copies of the above report for your files.

Sincerely,

Donald J. Mangold, Ph.D.
Director, Department of Applied Chemistry and Chemical Engineering

APPROVED:

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19. KEY WORDS (Continue on reverse side if necessary and identify by block number)

Antiradiation compounds: WR 2721; hydrolytic and thermal properties; encapsulation; drug dosage forms, blood plasma assay.

20. ABSTRACT (Continue on reverse side if necessary and identify by block number)

This report contains results of the following studies:

1. Encapsulation of WR 2721 for use as oral dosage forms.
2. In vitro evaluation of the most promising samples.
3. Development of analytical methodology for the active species.

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SUMMARY

This report covers studies conducted on S-2-(3-aminopropylamino)ethylphosphorothioic acid, WR 2721, over the period January 16, 1982 through January 15, 1983 which includes portions of the second and third years of the contract. The most significant results obtained during this report period are the following:

1. A blood assay method has been developed for WR 2721 over the range of ≤1 μg/mL to >1000 μg/mL.

2. The assay method has been used successfully in two preliminary dosing experiments with beagle dogs. WR 2721 appears to have an initial short half-life in plasma.

3. An HPLC blood assay method for WR 1065 and WR 33278 appears feasible based upon early results.

4. WR 2721 can be reproducibly encapsulated to give good protection of the drug against acid hydrolysis and rapid release at pH 7.5.

FOREWORD

Citations of organizations and tradenames do not constitute an official Department of the Army endorsement or approval of the products or services of these organizations.

In conducting the research described in this report, the investigator(s) adhered to the "Guide for the Care and Use of Laboratory Animals," prepared by the Committee on Care and Use of Laboratory Animals of the Institute of Laboratory Animal Resources, National Research Council [DHEW Publication No. (NIH) 78-23, Revised 1978].

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I. STATEMENT OF PROBLEM

Over a period of several years, the U.S. Army Medical Research and Development Command has been actively pursuing the development of a drug or combination of drugs which could be taken by military personnel for protection from the effects of the ionizing radiations from a nuclear weapons attack. Several chemical compounds (when dosed intravenously in animal studies) were found to be promising, in particular, the phosphorothioates. When administered IV the best of these materials, S-2-(3-aminopropylamino)ethylphosphorothioic acid (WR 2721), has been shown to protect mice, dogs, and rhesus monkeys against x-ray and/or gamma radiation and to protect mice against neutron radiation. However, oral dosing of WR 2721 failed to protect either dogs or monkeys and produced vomiting in dogs.

In an attempt to explain the lack of activity following oral administration, it has been postulated that WR 2721 is readily hydrolyzed to the mercaptan in the stomach of the animal species and that the mercaptan is poorly absorbed. (Credence has been given to this hypothesis as a result of studies completed during the first year of this program which showed that WR 2721 was readily hydrolyzed at a pH of 1.0). If such is the case, then it would be appropriate to protect WR 2721 with an enteric coating for passage through the stomach. One convenient method of applying such a coating is microencapsulation, a process which may subject the drug to elevated temperatures. Therefore, prior to undertaking any microencapsulation studies, adequate thermal stability of WR 2721 must be established.

During the previous term of the contract, WR 2721 was shown to be thermally stable and was successfully encapsulated as microspheres and as microcapsules. Several different matrices were developed which protected the drug from acid hydrolysis yet would release it in solutions of pH 7.5. These formulations appear promising as the initial candidates for oral dosing studies. However, prior to these studies a blood plasma assay for WR 2721 had to be developed. During this current term of the program an assay was established such that animal dosing studies can proceed.

II. BACKGROUND

During the period covered by the first and second annual reports under this contract, investigations were conducted which demonstrated the following:

- the hydrolytic instability of WR 2721 under acidic conditions but stability under alkaline conditions;
- the thermal stability of WR 2721 when heated at 60°C under nitrogen for at least one hour;
- the successful encapsulation of WR 2721 in a variety of glycerides, fatty acids and paraffins and mixtures thereof;
- the stability of WR 2721 in certain encapsulated products in pH 1.0 solutions at 37°C for 1.5 hours;
- the release of WR 2721 from certain of the promising encapsulated forms at pH 7.5 (37°C for 2 hours) using a buffered solution or synthetic intestinal fluid;
- the ability to directly assay microcapsules and some buffered solutions for WR 2721 using an HPLC procedure developed at SwRI;
- the ability to analyze for WR 2721 present in synthetic intestinal fluid using an alternate procedure which removes interferences in this system.

These results continue to justify the approach taken; that is, the development of an acceptable oral dosage form by encapsulation of the drug with enteric-type coatings which protect it from acid hydrolysis during passage through the stomach then release the drug in the intestinal tract.

In order to develop and evaluate such a dosage form, the reported studies were undertaken.

III. EXPERIMENTAL

A. Analytical Methods Development

1. Apparatus

a. HPLC Unit A

A Waters Associates Model 244 HPLC fitted with a Rheodyne 7125 injector, RCM-100 Radial Compression Module and Model 420 AC fluorescence detector comprised the flow system of Unit A. Excitation and emission filters were 395 and 460 nm, respectively. Peak integration was accomplished using a Waters Associates ''Data Module''.

b. HPLC Unit B

An IBM Model LC533 HPLC was fitted with an RCM-100, Model 420 AC fluorescence detector and a Houston Instruments strip-chart recorder to form HPLC Unit B.

c. Solvents

Organic solvents were Burdick and Jackson ''distilled-in-glass'' grade. Water was purified with a Millipore Milli RO4 purification system.

d. Reagents

All reagents were ACS reagent grade. Alkyl amines were purchased from Aldrich Chemical Company.

B. In Vivo Studies

Beagle and mongrel dogs were dosed IV with WR 2721 in pilot investigations. The initial study with a mature mongrel dog was at a level of 135 mg/kg of WR 2721. Two subsequent studies have been completed at the 150 mg/kg level with a mature male beagle dog and an additional dosing study has just been completed but plasma assay data is pending. Blood samples were withdrawn from the mongrel dog at zero time and at 20, 40 sec, 1, 2, 3, 6, 9, 12, 15, 22, 30, 45, 60, 90, 120, 150, 180, 210, 300, and 360 min post infusion. (Dose infusion over 20 sec; time started at 10 sec).

Blood samples for the beagle dog were withdrawn at 1, 2, 3, 6, 9, 12, 15, 22, 30, 45, 60, 90, 120, 150, 180, 210, 300 and 360 min post infusion. Dose infusion over 2 min; time started after infusion. Blood plasma was separated from the samples and analyzed by the HPLC procedures described in Section IV.

Additional details concerning the procedures are presented in Appendix A.

C. Microencapsulation

1. Materials

a. Description and Source

All materials used in the encapsulation formulations were standard food-grade, and their sources are listed in Appendix B.

b. Purity

The glycerides, fatty acids, fatty alcohols, and paraffin wax listed in Appendix B were used as received.

WR 2721 was obtained as the trihydrate from WRAIR and was used as received except as noted, where the particle size was reduced by milling with a mortar and pestle. The material as originally received was labeled "WR 2721 AU BJ 09506-AJ-68-2." Analysis was reported as 79.2 percent WR 2721, <0.5 percent mercaptan and disulfude, 19.7 percent H_2O and approximately 0.4 percent unidentified impurities.

2. Procedures

The centrifugal extrusion process as described in the second annual report for the project was used to prepare the microspheres and microcapsules described.

A hot-melt system was used to prepare microcapsules and microspheres. All reservoir, pump and feed lines were separately wrapped with heating tapes individually controlled by variable transformers. Head temperature was controlled by a Lepel high frequency induction heater. Nozzle size, head rotational speed, and feed rates were optimized to produce microcapsules and microspheres mainly in the 500 to 710 μ size range. Final sizing was done using standard stainless steel sieves.

3. Evaluation

a. Hydrolytic Stability

Hydrolytic stability of microspheres and microcapsules was determined by subjecting samples to HCl/KCl buffer solutions at pH levels of approximately 1 and 3 at 37°C for 90 minutes using the standard rotating bottle method described in the Annual Report dated January 1982.

b. In Vitro Release Rates

Release rates of microspheres and microcapsules were determined by subjecting samples to Tris buffer [aqueous solution of HCl/tris(hydroxymethyl)-aminomethane] and to synthetic intestinal fluid at approximately pH 7.5 at 37°C for periods of 0.25, 0.50, 1, 2, 3, 5, and 8 hours as described in the Annual Report dated January 1982.

IV. RESULTS

A. Analytical Methods Development

1. Background Work - Derivatization of WR 2721 (SFRE)

During this reporting period, work was continued on the development of an assay for the detection of WR 2721 and its metabolites in plasma utilizing derivatization with fluorescamine and subsequent separation and quantitation by HPLC. The derivatization reaction and fluorescent properties of the derivatives of WR 2721, the mercaptan and disulfide, were discussed in detail in Annual Report dated January 1982.

It was previously determined that the fluorescence emission of the derivatives as a function of concentration of drug (or metabolite) exhibits sharp changes in slope, indicative of inner-filter quenching (Annual Report dated January 1982). There are published reports that this type of concentration-dependent departure from linearity can be improved by shifting the excitation wavelength from 390 nm (398 nm for WR 2721) to 340 nm. Therefore, the effect of this shift in excitation wavelength upon the emission response as a function of concentration has been evaluated. It was found that, although this change may improve the linearity of emission, it also diminishes the emission so markedly that significant loss of assay sensitivity would result.

In a continuation of the effort to explore the lower limits of detectability of the derivatized WR 2721 and metabolites, it has been determined that less than 200 picomoles of derivatized WR 2721 or mercaptan metabolite can be detected on reverse phase thin layer chromatographic plates employing long wavelength UV light. Although low concentrations of the fluorescamine derivatives of drug and both metabolites could be detected easily in these thin layer systems, all three compounds exhibited essentially the same relative mobility in a range of mobile phase compositions of the type usually employed for reverse phase HPLC columns. In an effort to resolve the drug and metabolite derivatives, a number of potential mobile phase compositions were explored. In these preliminary experiments, the best resolution appeared to be provided by solvent systems of the type used for separation of amphipathic molecules such as phospholipids.

It appears practicable to derivatize WR 2721 and its metabolites with fluorescamine by addition of fluorescamine solution directly to plasma. This offers many advantages with respect to speed and simplicity of assay; there are, however, a number of potential interferences and artifacts in such a direct approach which must be evaluated.

In experiments in which WR 2721 was added to plasma and subsequently analyzed on TLC, significantly higher proportions of the RSH form were observed than when the plasma was omitted (an equivalent volume of buffer being substituted). For these experiments WR 2721 was added to chilled canine plasma, the fluorescamine derivatization was performed within minutes, the samples were stored in an ice bath and aliquots of such samples were removed for either direct separation on TLC or solvent (chloroform or ether) extraction and subsequent TLC separation. Possible reasons for this increase in RSH which were considered included: (1)

WR 2721 was being rapidly hydrolyzed by plasma, even though plasma/drug samples were never allowed to warm above 0°C - conditions under which no enzymatic activity should be encountered; (2) WR 2721 was being hydrolyzed during the derivatization reaction in the presence of plasma to a much larger extent than in buffer; or (3) significantly more mercaptan derivative was being extracted than WR 2721 derivative.

It is not likely that WR 2721 will be cleaved enzymatically at ice bath temperatures. The plasma enzyme reported to be responsible for the cleavage of the phosphate group from WR 2721 is acid phosphatase[1] although cellular elements of the blood may possess additional enzymatic activities capable of this cleavage.[1]

In consideration of the second possibility, the technique for derivative formation was modified somewhat to reduce the likelihood of hydrolysis. Modifications included a change in buffer composition, a change in reaction protocol, and a change in solvent for fluorescamine. Fluorescamine was initially dissolved in acetone, but dioxane was substituted because it is less volatile and probably less reactive under the slightly basic conditions employed. The modified technique employs Clark and Lubs borate buffer, which is 0.1M KCl plus 0.1M boric acid, buffered to the desired pH with 0.1N NaOH. WR 2721 is dissolved in this buffer at pH 10.0 to obtain a concentrated stock standard solution of 10 µg/µL. Derivatization was accomplished by rapid mixing of 2 µL (20 µg) of this stock solution with 100 µL of borate buffer, pH 7.6, followed by rapid addition of 300 µL of fluorescamine in dioxane (12 to 15 mg/50 mL) while vortexing vigorously; vortexing was continued for at least 30 seconds. The derivative thus formed was kept on ice until assayed.

Concomitant with these changes, a new pH profile for WR 2721 derivative fluorescence as a function of pH of derivatization reaction mixture was measured. The pH profile obtained for the derivatization of WR 2721 by this modified method is shown in Figure 1. This method would appear to enhance the extent of the derivatization reaction, although the pH profile cannot be compared with that reported earlier because the results were obtained under different conditions with respect to reactant concentrations and buffer composition. The original buffer contained ammonium ion, which, although reported not to interfere in the reaction between fluorescamine and primary amines, may have been responsible for some anomalous results. Both pH profiles show the same narrow range of optimal pH - approximately 7.5 to 8.0 - and the same rapid decrease in emission with increasing pH.

The pH profile for the derivatization of the metabolites RSH and RSSR under these conditions were also obtained, and these are shown in Figure 2. While the curve shape is the same as for WR 2721, the increase in emission response is approximately three-fold greater for RSH and more than two-fold greater for RSSR than for WR 2721. The reason for this difference in response is not known at this time. Since the three pH profiles were obtained with nominally equivalent weights of WR 2721, RSH, and RSSR (instead of equal molar concentrations) and a standard concentration of fluorescamine, it was decided to explore the effects of varying the molar ratios of fluorescamine and WR 2721/metabolite upon fluorescence emission obtained. Results of the first several experiments are shown in Figure 3; concentration of WR 2721 was held constant at 90 µmolar (20 µg/mL) while the concentration of fluorescamine was varied from approximately 40 to 2200 µmolar.

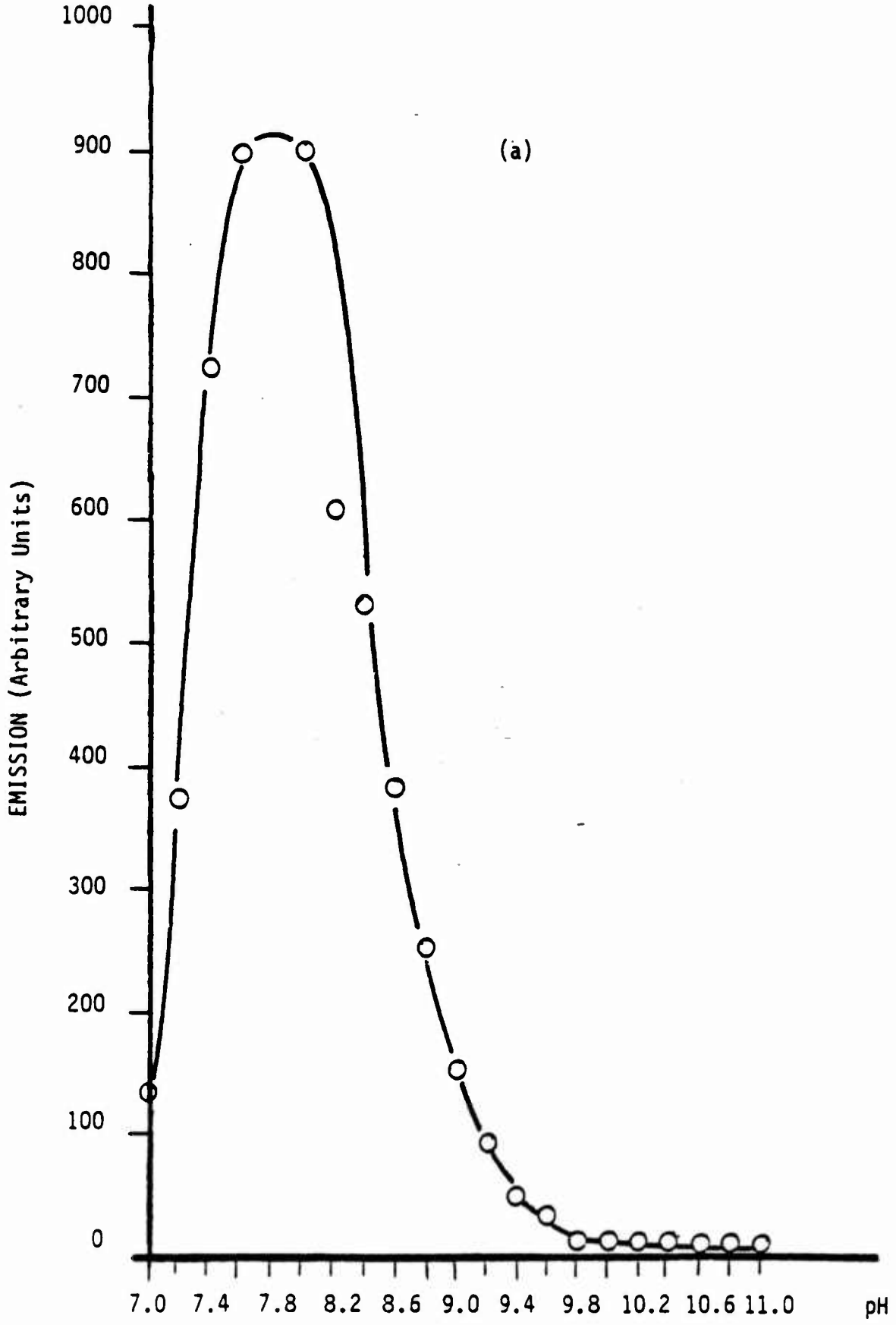

Figure 1. pH Profile Obtained for Derivatization of WR 2721.

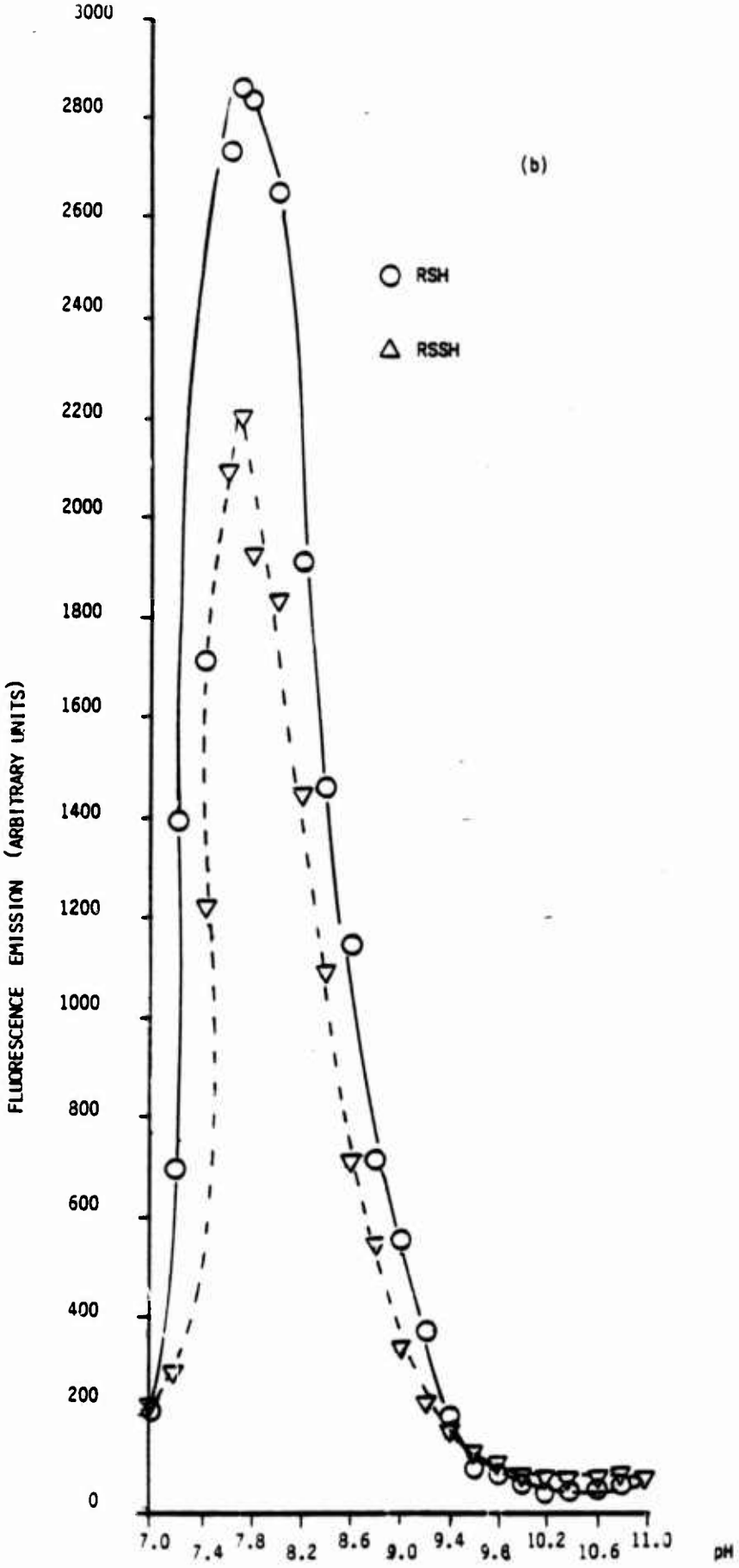

Figure 2. pH Profile Obtained for Derivatization of RSH and RSSR.

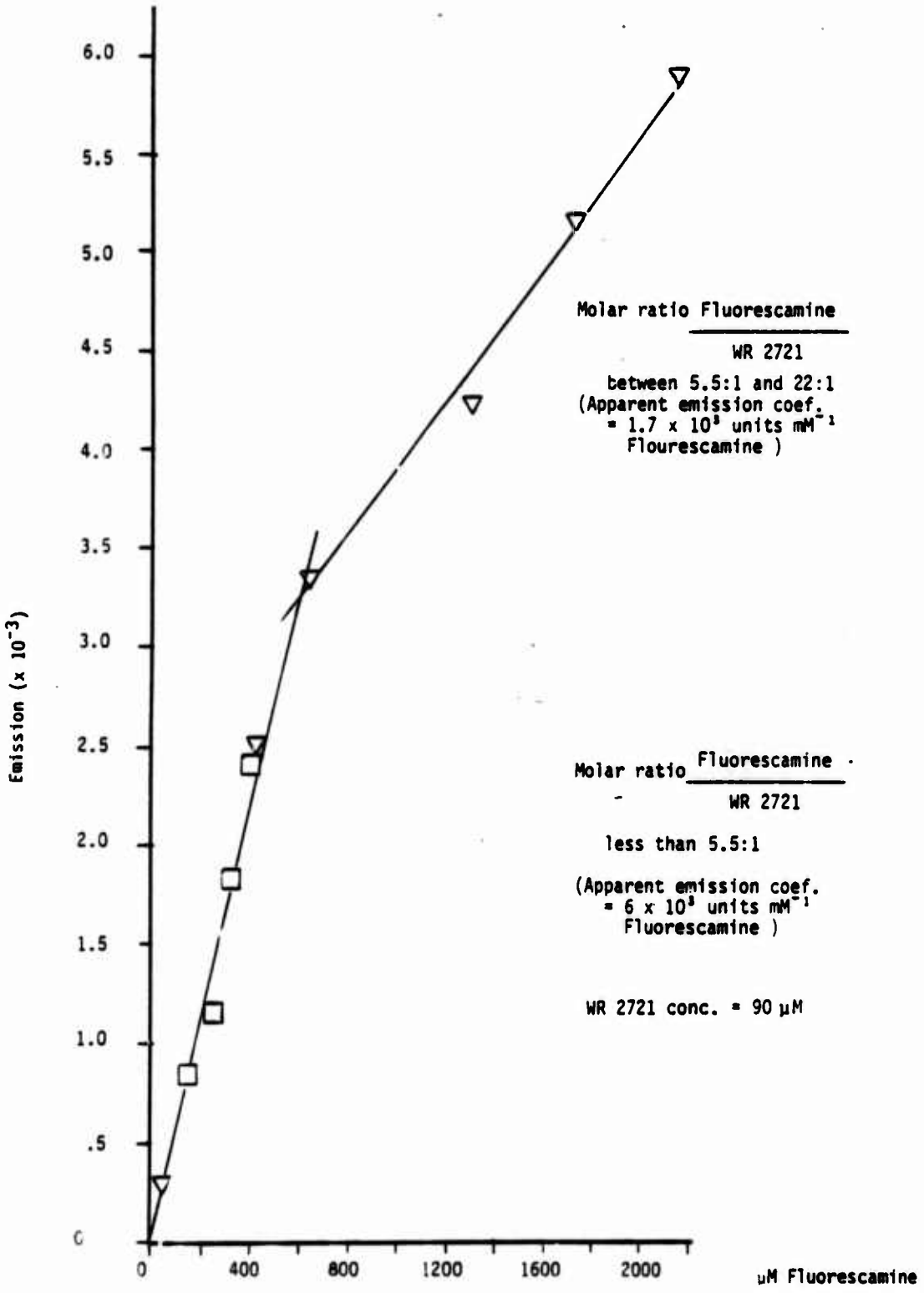

Figure 3. Effects of Varying the Molar Ratios of Fluorescamine and WR 2721/Metabolite Upon Fluorescence Emission.

From the slopes of the lines shown in Figure 3, it is possible to estimate two different values of an apparent coefficient of fluorescence emission for the WR 2721-fluorescamine adduct depending upon the range of molar ratios of the two reagents. At fluorescamine to WR 2721 molar ratios below 4.4:1 (the normal working range), the apparent emission coefficient is approximately 6,000 (6×10^3) fluorescence units nm^{-1} fluorescamine. At higher concentrations of fluorescamine - i.e., molar ratios from 5.5 - 22:1 of fluorescamine to WR 2721, the apparent emission coefficient decreases to a value of 1,700 (1.7×10^3) units nm^{-1}.

It is likely that extraction of samples of WR 2721 with chloroform or ether was resulting in a preferential concentration of RSH form relative to WR 2721 in the extract. In experiments in which the extraction capacity of a number of different solvents was compared by HPLC quantitation (Table 1), it was seen that hydrophobic solvents tended to extract much more of the small amount of RSH form originally present than of the WR 2721. This effect was not favorable for the recovery and subsequent quantitation of WR 2721 in plasma; however, this preferential solubility of RSH over WR 2721 in hydrophobic solvents may offer a relatively simple approach to the assay of RSH in plasma.

In summary, the fluorescamine derivative of WR 2721 and the mercaptan and disulfide metabolites may be formed in plasma by the addition of fluorescamine in solution to plasma with rapid mixing (vortexing). Fluorescamine reacts with primary amino groups under basic conditions almost instantaneously to form highly fluorescent products. The fluorophores formed are relatively stable and there is no interference from reactions with other active groups such as secondary amino or sulfhydryl groups because these products are not fluorescent; excess reagent is also hydrolyzed immediately to non-fluorescent products. Interfering substances are endogenous plasma components containing free amino groups such as amino acids, peptides, proteins, primary amines, etc. Plasma containing WR 2721 may be derivatized and loaded directly onto an HPLC column for separation, detection, and quantitation of fluorophores. However, for routine work, application of physiological fluids onto columns could introduce a variable because column retentive capacity and separating ability could be altered significantly as successive samples are analyzed. It has been determined that it is possible to derivatize added WR 2721 in dog plasma and subsequently extract the derivative into an immiscible organic phase - chloroform. When this $CHCl_3$ extract was chromatographed on reverse phase TLC, three major spots were obtained, one of which appeared to migrate similarly to standard WR 2721 and mercaptan fluorescamine derivatives.

2. HPLC of WR 2721 and Its Metabolites

a. PAC Column - Method I

The polar amino/cyano column (PAC, Whatman, Inc.) used for the direct assay of WR 2721 in microspheres was found to separate the fluorescamine derivatives of WR 2721 and WR 1065 under the following conditions:

TABLE 1. EXTRACTION OF FLUORESCAMINE DERIVATIVES OF WR 2721 AND ITS MERCAPTAN FROM AQUEOUS SYSTEMS

Solvent System[a]	Peak Area[b]					Volume of Organic Layer, uL	Relative Extraction Efficiency[d]
	Mercapten Derivative	$RSPO_3H_2$ Derivative	Total	Corrected[c] Total	Ratio Mercaptan/$RSPO_3H_2$		
isopropyl ether	2017	3305	6919	8995	39/61	650	0.19
ethyl acetate	11664	12934	24598	36897	47/53	725	0.99
toluene	11668	13267	24935	34909	47/53	700	0.84
ethyl ether	10247	11669	21916	28491	47/53	650	0.76
chloroform	13868	5892	19760	29640	70/30	750	0.80
heptane/trioctylamine (3:2)	6680	2105	8735	10045	76/24	575	0.27
chloroform/methanol (4:1)	6377	9390	20497	938	40/60	650	0.55
n-butanol/trioctylamine (1:1)	816	ND	816	938	—	575	0.03
n-butanol/benzene (5:1)	5812	29693	35505	37280	16/84	525	1.00

ND - none detected

a. Extraction of 500 uL of water/acetone (1:1) solution of derivatives with 500 uL of solvent; original ratio of mercaptan:WR 2721 was 3:97.
b. HPLC analysis.
c. Corrected to 500 uL of organic phase.
d. WR 2721 plus WR 1065, relative to n-BuOH/benzene (5:1).

Column:	Whatman PAC (10 µm), 250 mm x 4.6 mm stainless steel
Mobile Phase:	Acetonitrile/water (400), 0.015 phosphate buffer, pH = 7.2
Flow Rate:	1.0 mL/minute
Detector:	Waters Associates Model 420 Fluorescence Detector - Excitation filter 395 nm - Emission filter 460 nm

Elution times were 3.0 and 3.9 minutes for WR 1065 and WR 2721, respectively. A representative HPLC trace is shown in Figure 4.

This system was used to determine extraction efficiencies of the derivatives when present in aqueous systems. In these experiments 500 µL of water/acetone (1:1) spiked to 67 ng/µL with a 97:3 mixture of WR 2721/WR 1065 was shaken with an equal volume of the solvent system and, after phase separation, the volumes of the layers were measured and aliquots were injected onto the HPLC column. Results of the experiment are summarized in Table 1. In each solvent system the ratio of WR 1065 to WR 2721 was much higher than the starting ratio, implying that the less polar mercaptan derivative partitions better into the organic phase and/or the WR 2721 derivative is hydrolyzing to the former during the experiment. The solvent system n-butyl alcohol/benzene (5:1) was retested but different results were obtained. When an aliquot of the lower (aqueous) phase was injected onto the HPLC column, the chromatogram in Figure 5 was produced. This shows a two-fold increase in the WR 2721 peak area and a smaller (1.5X) increase in the mercaptan peak area, both of which are attributable to the concentration effect (resulting volume of aqueous phase < volume of organic phase). Analysis of the upper (organic) layer in the same way gave no significant response for either material (Figure 6). When this experiment was carried out on canine plasma spiked to the same level with WR 2721, peaks for both compounds were detected (Figure 7). Recovery of WR 2721 was 82% while the apparent mercaptan level was greatly increased. The latter result is explainable in part by interferences present in the plasma (Figure 8) which were not separated under these chromatographic conditions.

After correction for this interference, the mercaptan recovery was still greater than 100% but accountability of WR 2721 plus mercaptan was good (108%). These results indicate that some conversion of WR 2721 to its mercaptan was occurring. This cleavage could occur to WR 2721, its derivative, or to both.

A sample of canine plasma spiked with WR 2721 was heated in a boiling water bath for several minutes, then cooled, derivatized, extracted, and analyzed as above (Figure 9). The WR 2721 peak was reduced by 17,770 area units (10%) and the mercaptan peak increased by 6,440 area units (13%). This implies that some hydrolysis occurred during the heating. It was hoped that the heat treatment would deactivate enzymes responsible for WR 2721 cleavage.

Figure 4. HPLC Trace of Fluorescamine Derivatives of WR 2721 and WR 1065 Obtained Using PAC Column.

INJECT

APR. 15, 1982 15:28:46 CHART 0.50 CM/MIN FLOW 1.00 ML/MIN
PRESSURE 700.0 DETECTOR 460/395
RUN #6 CALC #0
COLUMN SOLVENT OPR ID: 12

EXTERNAL STANDARD QUANTITATION

PEAK#	AMOUNT	RT	EXP RT	AREA		RF
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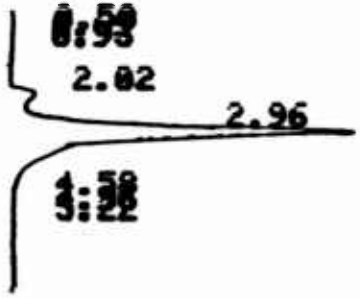
	189.80900	2.56		189809	F	0.000000E0
	1585.99000	3.01		1585991	F	0.000000E0
	80907.30000	3.89		80907917	F	0.000000E0
	2232.07000	5.14		2232077	F	0.000000E0
	1280.42000	5.62		1280422	F	0.000000E0
	4.98800	6.13		4988	F	0.000000E0
	690.70700	6.42		690707	F	0.000000E0
	4799.33000	7.22		4799356	HL	0.000000E0
TOTAL	91812.90000					

Figure 5. HPLC Trace of Aqueous Extract Fluorescamine Derivative of WR 2721 Obtained Using PAC Column.

Figure 6. HPLC Trace of Organic Extract (Butanol/Benzene) of Fluorescamine Derivative of WR 2721 Obtained Using PAC Column.

Figure 7. HPLC Trace of Canine Blood Plasma Spiked with WR 2721 and Derivatized with Fluorescamine.

INJECT



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COLUMN SOLVENT OPR ID: 12

EXTERNAL STANDARD QUANTITATION

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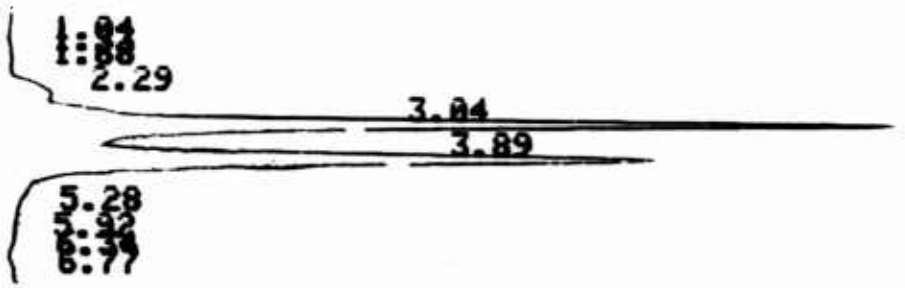
Figure 8. HPLC Trace of Canine Plasma Derivatized with Fluorescamine.



APR. 20, 1982 14:21:55 CHART 0.50 CM/MIN FLOW 1.00 ML/MIN
PRESSURE 800.0 DETECTOR 460/395
RUN #4 CALC #0
COLUMN SOLVENT OFR ID: 12

EXTERNAL STANDARD QUANTITATION

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	273.72700	1.33		273727	F	0.000000E0
	195.30400	1.68		195304	F	0.000000E0
	3950.26000	2.29		3950275	F	0.000000E0
	54282.40000	3.04		54282737	F	0.000000E0
	48751.90000	3.89		48752242	F	0.000000E0
	1382.50000	5.28		1382510	F	0.000000E0
	24.31300	5.92		24313	F	0.000000E0
	338.76900	6.34		338769	F	0.000000E0
	725.22400	6.77		725224	HL	0.000000E0
TOTAL	110109.00000					

Figure 9. HPLC Trace of Canine Plasma Spiked with WR 2721 Then Heated in a Boiling Water Bath.

b. Radial Compression Module - Method II, No Internal Standard

It was shown in earlier work on this program that WR 2721 was essentially unretained on a C-18 reverse phase column and eluted at or near the void volume of the column when a variety of organic/water mixtures and ratios were used as the mobile phase. Derivatization with fluorescamine and the addition of ion-pairing reagents[3] convert the polar WR 2721 molecule into a species which is retained on a number of reverse phase columns using a variety of mobile phase combinations.

Until recently the system found most successful for the separation of WR 2721 from plasma consisted of a Waters Associates Radial Compression Module (RCM-100) fitted with a C-18 reverse phase cartridge and a 22/78 CH_3CN/water mobile phase which was 0.01M in dibutylammonium phosphate (DBAP), pH = 2.8. This system separated the fluorescamine derivative of WR 2721 from all but the smallest interference present in plasmas taken from beagle dogs which serve as animal models. Similar separations were obtained on other C-18 columns, but the RCM-100 gave superior resolution, better peak shapes, lower back pressure and faster equilibration times than any other tested. Acetonitrile was superior to methanol, 2-propanol and tetrahydrofuran as the organic component of the mobile phase because better peak shapes and consequently better resolution was obtained. Resolution was very important in this separation because of the presence of a large number of peaks due to endogenous amines. Chromatographic conditions of this method - Method II - are summarized as follows:

Column:	Waters Associates RCM-100 with 5 μm C-18 cartridge (15 cm x 0.8 cm)
Mobile Phase:	Acetonitrile/water (22/78), 0.01M in dibutylammonium phosphate
Flow Rate:	2.0 mL/minute
Detector:	Waters Associates Model 420 AC Fluorescence Detector - Excitation 395 nm - Emission 460 nm
Guard Column:	Whatman guard column filled with pellicular C-18 packing

Under these conditions, the derivatized WR 2721 eluted from the column in approximately 14 minutes and the last peak was observed in <60 minutes. A representative chromatogram of a ''spiked'' sample of beagle plasma (80 μg WR 2721/mL) is shown in Figure 10. Comparison of Figure 10 with that obtained from ''unspiked'' beagle plasma (Figure 11) shows no major interferences from endogenous components. If necessary, total analysis time may be reduced to approximately 30 minutes by increasing flow rate to 4 mL/minute (see Figure 12).

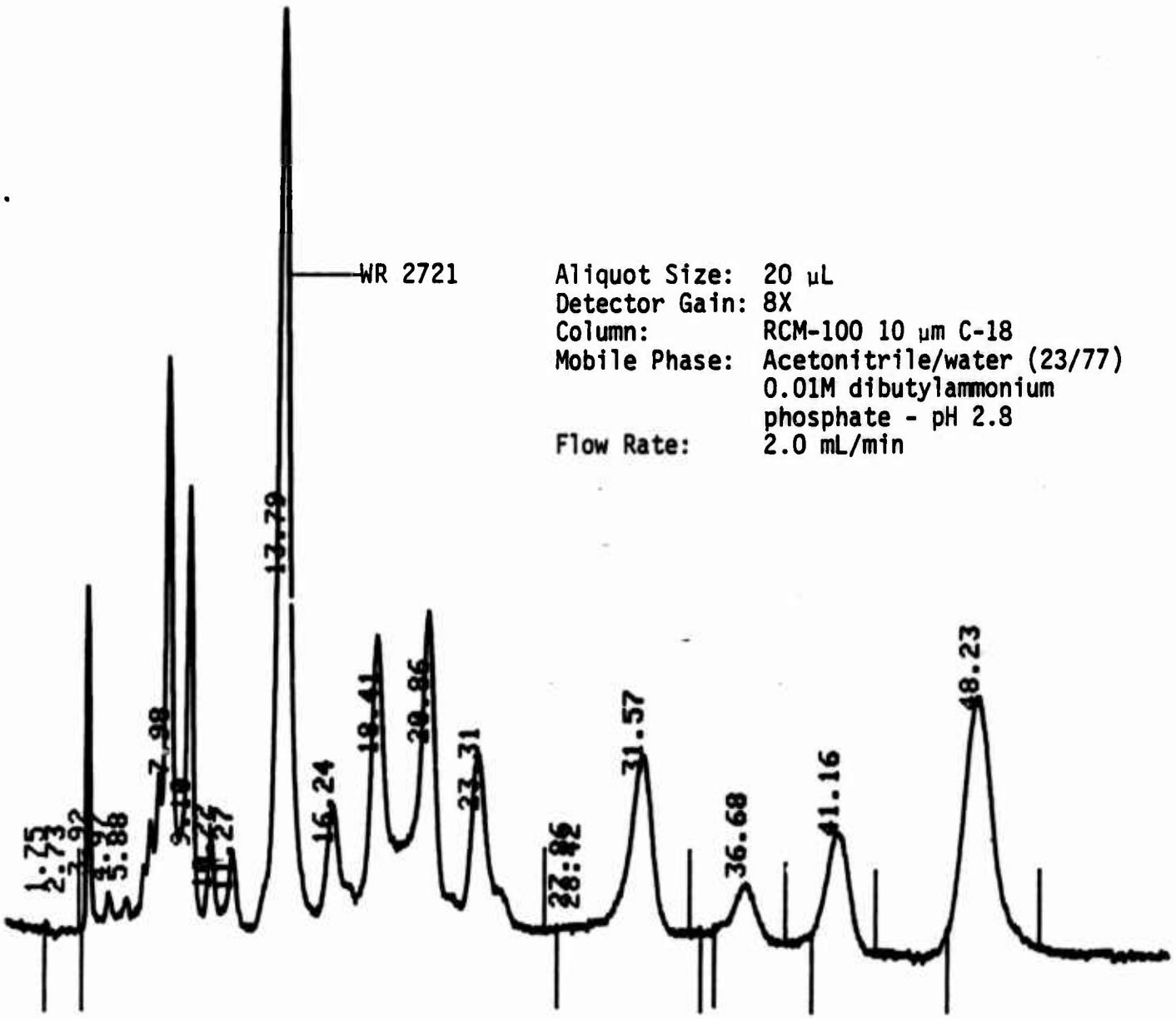


Figure 10. WR 2721 in Beagle Plasma (80 µg/mL), 10 µm C-18 Column.

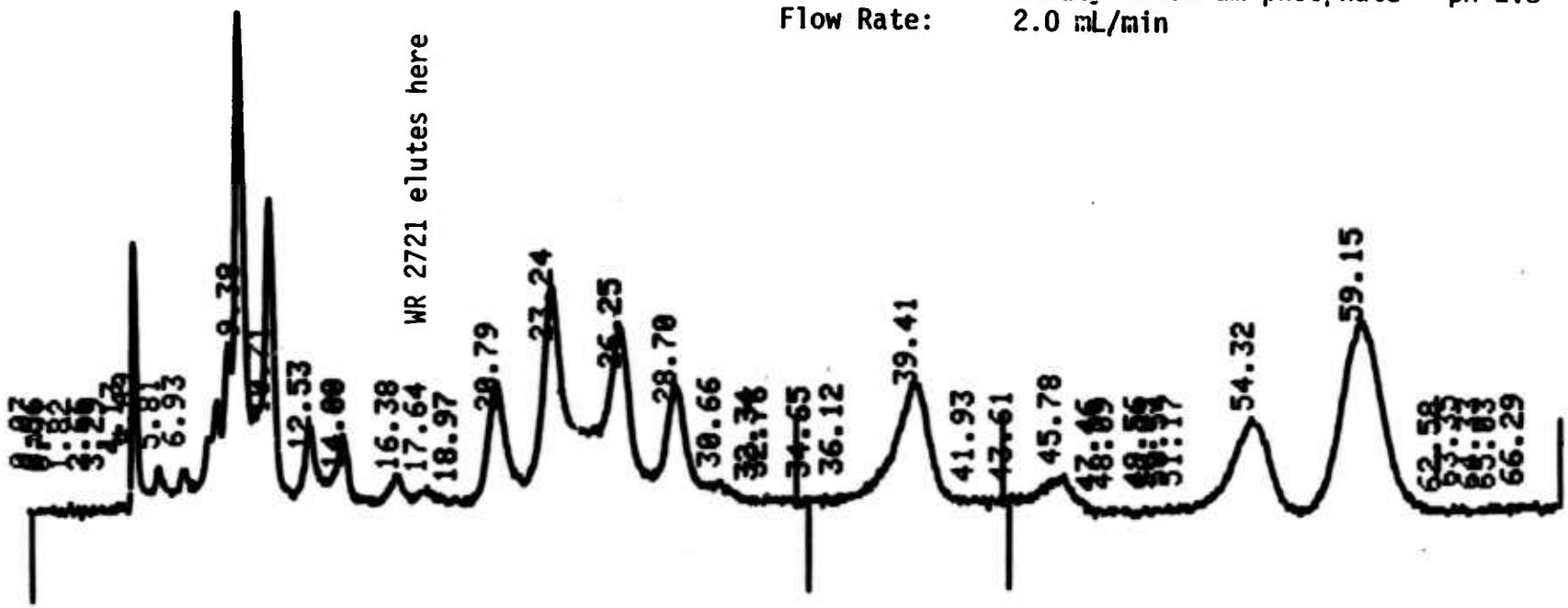


Figure 11. Beagle Plasma, 5 µm C-18 Column.

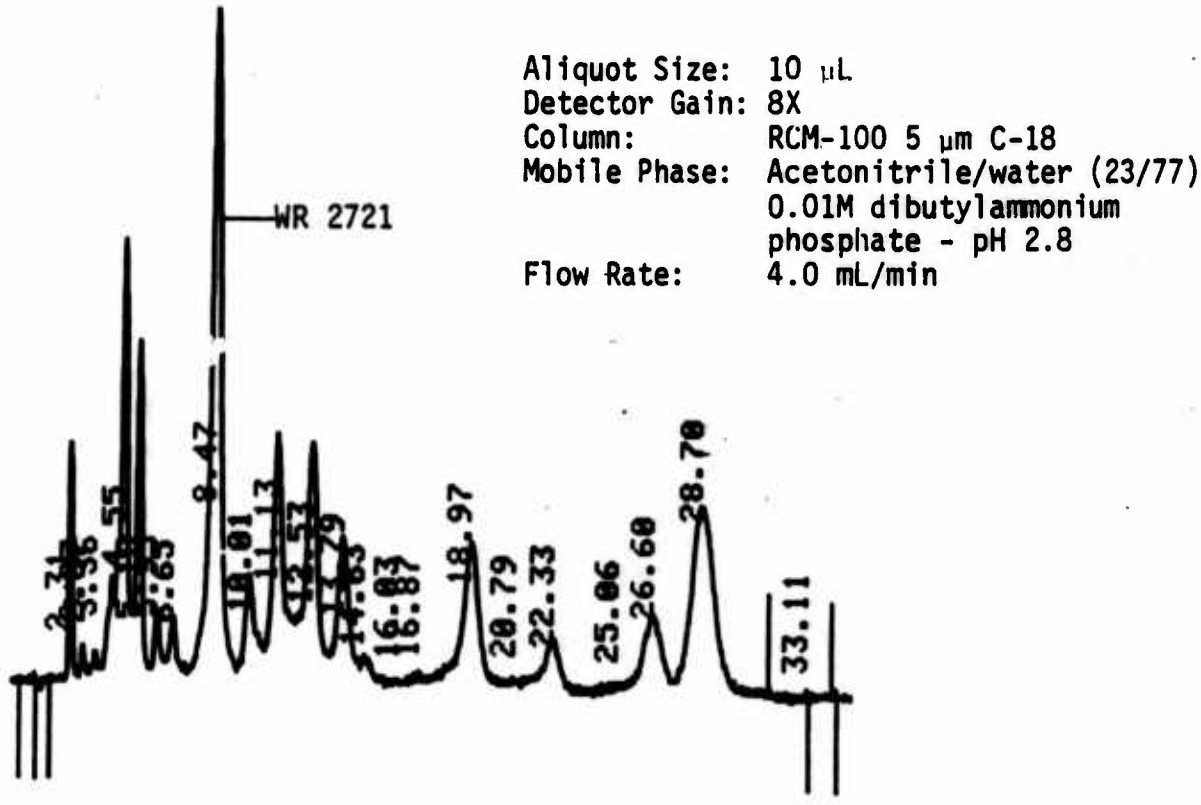


Figure 12. Beagle Plasma Spiked with WR 2721, 80 µg/mL, 5 µm C-18 Column.

Repeatability of the procedure at three levels (8, 80, and 792 μg/mL) was demonstrated by multiple analysis of samples of beagle plasma spiked with WR 2721. Results are summarized in Table 2.

TABLE 2. REPEATABILITY OF WR 2721 ASSAY

	WR 2721 Concentration		
	8 ug/mL	80 ug/mL	792 ug/mL
Repeatability[a]	8.3	9.5	7.0
Recovery[b]	64.3	90.3	95.0

a. Relative standard deviation $(s \times 100)/\bar{x}$.
b. Expressed as percent of assay of same level of WR 2721 present in pH 8 buffer.

Relative standard deviation was <10% at each level. Recoveries (relative to an external standard of WR 2721 in buffer solution) were >90% at the two higher levels. At 8 μg/mL 65% of the spiked material was recovered. Figure 13 graphically presents the results of analysis of varying concentrations of WR 2721 in plasma and in buffer solution. The plasma series was repeated the following day. Close correspondence at the same levels between plasma and buffer solutions was obtained. This indicates that an external standard could be used to estimate the amount of WR 2721 present in plasma at the time of analysis. Data for the plots in Figure 13 are given in Table 3. These values were obtained after a modification to the derivatization procedure. Previously, lack of repeatability had been a source of concern and had been attributed to binding of the added WR 2721 to plasma components. Addition of methanol, ethanol, or acetonitrile to the spiked plasma in attempts to denature it did not give significant improvement in repeatability or recovery. When the derivatizing reagent was added to spiked plasma in two portions, with a 30 s agitation period intervening, larger, more repeatable peaks for WR 2721 were immediately observed. It is theorized that the acetone solvent for fluorescamine serves to free bound WR 2721 and the second portion of reagent derivatizes the freed material. This explanation is based in part on the rapid decomposition of fluorescamine in aqueous media. The derivatization procedure is as follows:

1) Place 100 μL plasma in a suitable vial and add 200 μL of pH 7.8 borate buffer.

2) Agitate this mixture on a vortex mixer and add 200 μL of fluorescamine in acetone solution (5 mg/mL) to the mixture under agitation.

3) Continue the agitation for 20 to 30 s, repeat Step 2, and agitate an additional 20 to 30 s.

4) Centrifuge sample and inject an aliquot of the supernatant (usually 20 to 50 μL) onto the HPLC column.

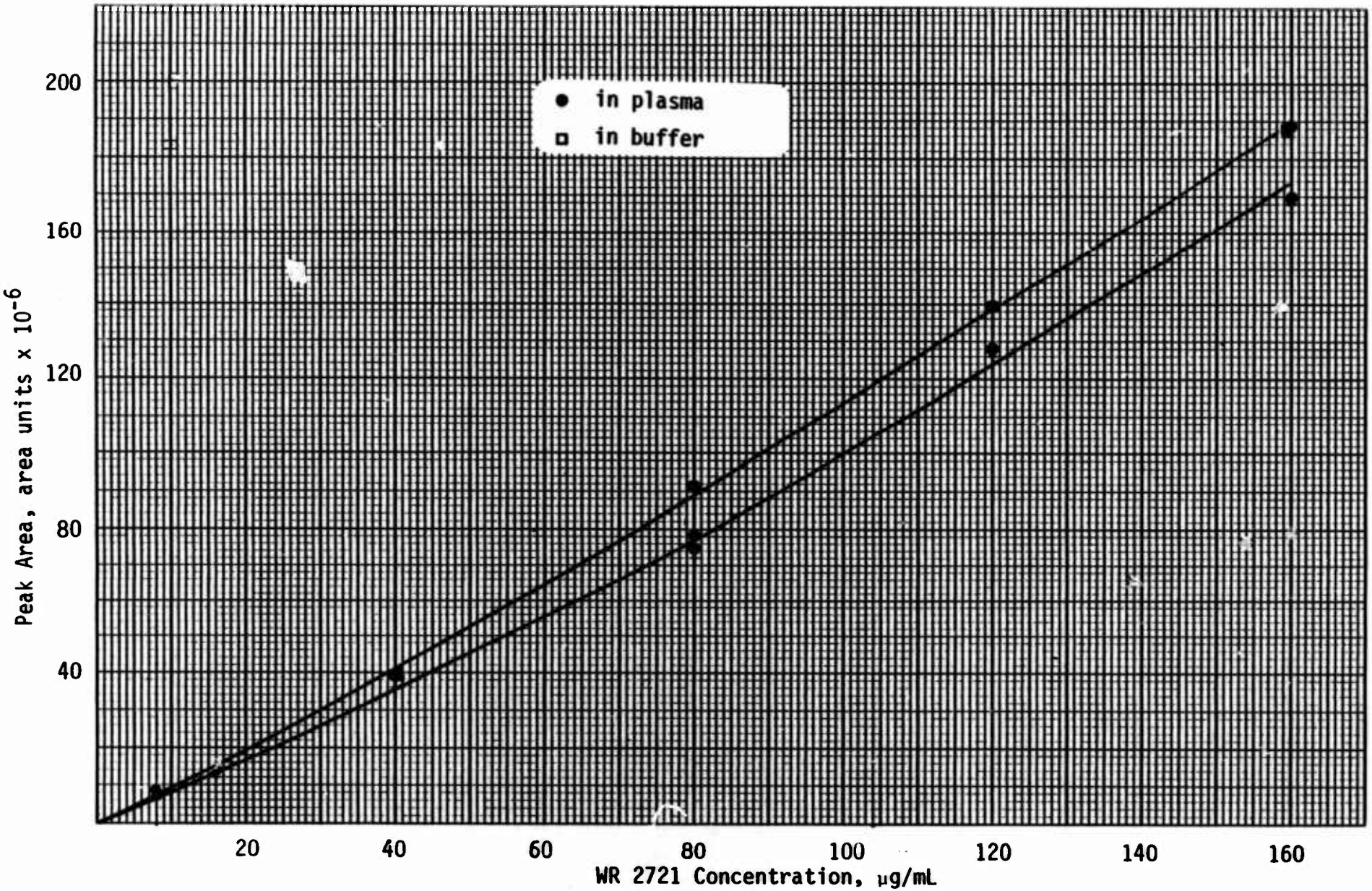


Figure 13. HPLC Standard Curves of WR 2721 in Beagle Plasma and in Buffer.

TABLE 3. HPLC ANALYSIS OF VARYING AMOUNTS OF WR 2721 IN BEAGLE PLASMA AND IN BUFFER

WR 2721 Concentration µg/mL	Peak Area of Fluorescamine Derivative area units x 10^{-6}		
	In Buffer[a]	In Plasma[a]	In Plasma[b]
8	--	--	8.6
16	--	13.9	--
40	39.7	39.5	39.4
80	91.4	78.3	74.3
120	139.1	128.1	--
160	188.4	188.5	170.5

a. Analyzed July 13, 1982.
b. Analyzed July 14, 1982.

Note that no extraction or precolumn cleanup was used. Because of the resolving power of the column, WR 2721 (as its fluorescamine derivative) was separated from interferences under conditions which do not elute approximately one-half of the total of fluorescent species, i.e., the less polar components. Retention of these materials posed no problem. The sensitivity of the analysis was high, consequently the mass injected on the column was low. The only effect was to slightly shorten elution times over the course of the day's analyses. Flushing the column with 100 to 150 mL of methanol/water (70/30) at the end of the day completely restored it to original conditions.

A search was carried out to find a compound suitable for use as an internal standard in Method II. Requirements were that the compound be fluorescent or be capable of being converted to a fluorescent derivative and must elute in a region of the chromatogram free from interferences. To date 37 compounds have been screened but a suitable standard has not been found. Most compounds tested do not elute from the column or have excessive elution times. A homolog of WR 2721 was found to chromatograph satisfactorily but was unstable in buffer solution, relative to WR 2721. This compound, WR 149023, was also tested for stability of its fluorescamine derivative at 23°C. Its decomposition rate was essentially the same as that of the corresponding derivative of WR 2721, having a half-life of 46 minutes.

Three esters of serine were also tested, the methyl ester which was purchased and the ethyl and isopropyl esters which were synthesized. The isopropyl ester had suitable chromatographic characteristics but was unstable in aqueous solution, undergoing hydrolysis. A list of compounds screened is presented in Table 4.

TABLE 4. COMPOUNDS INVESTIGATED AS POSSIBLE INTERNAL STANDARDS

N-acetyl-l-glutamine	(2-aminoethoxy)ethanol
dl-aspartic acid	3-amino-1-propanol
histidyl histidine	dl-1-amino-2-propanol
glycyl glycyl glycyl glycine	ethanolamine
polyglycine	2-amino-2-ethyl-1,3-propanediol
poly-l-tyrosine	2-amino-2-methyl-1-propanol
l-histidine	3-methoxy-4-hydroxyphenyl ethylamine
dl-serine	alanyl glycyl clycine
dl-serine methyl ester	WR 74172
dl-serine ethyl ester	WR 80855
dl-serine isopropyl ester	WR 149023
l-tyrosine	WR 172730
l-tryptophane	WR 1490
l-methionine	WR 4470
l-cysteine	WR 1961
3-hydroxytyramine	WR 3311
l-β-3,4-dihydroxyphenylalanine	WR 2819
2-amino-1-butanol	WR 638
2-amino-6-chloro-4-pyrimidinol monohydrate	

c. Radial Compression Module - Method III. ^{14}C-Labeled WR 2721 as Internal Standard

Because a suitable internal standard for the HPLC analysis of WR 2721 in plasma has yet to be found, it was decided to characterize a method of analysis using the HPLC techniques already described but having ^{14}C-radiolabeled WR 2721 serve as a standard. Therefore, radiolabeled WR 2721 was added to plasma samples immediately after they were drawn and upon analysis the column effluent was collected and counted in order to determine recovery. Excellent precision and accuracy values were obtained over the approximate range of 3-1100 µg/mL using this procedure.

The precision of the method over the entire working range was determined by the analysis of replicate spiked samples. In Table 5 the concentration, number of replicates analyzed and the relative standard deviation (RSD) for each data set are presented. The average RSD for the method was 6.6%.

TABLE 5. PRECISION OF WR 2721 ANALYTICAL METHOD

WR 2721 Concentration, µg/mL	Number of Replicates Analyzed, N	RSD (%)
1112	5	9.1
112	5	5.3
11.9	5	5.6
6.3	5	5.1
3.0	5	7.8

Accuracy of the method for plasma concentration ranging from 3 to 1112 μg/mL was determined by the analysis of blind spiked plasma samples. spike levels and percent recovery are presented in Table 6. Recovery over the entire method range was within acceptable limits.

TABLE 6. SPIKE LEVELS AND PERCENT RECOVERY

Spike Level, μg/mL	Spike Recovery Percent
2.92	89
7.19	121
10.41	117
3.99	100
1.85	92
1112	91
	Mean = 104

d. Radial Compression Module - Method IV

It was found that a peak due to an endogenous material in beagle plasma coeluted with WR 2721 under the conditions of Methods II and III. In the development of the methods it had gone undetected, partly because of its small size (area = 0.5-1 μg/mL of WR 2721) and partly because the detector used at that time had a sensitivity which was ten times less than that of the one currently in use. Modifications to the chromatographic system were made to separate WR 2721 from this interference. Changes in pH and ion-pairing reagent was made to the mobile phase and standards of beagle plasma containing low levels of WR 2721 were injected. Variations in pH outside the 2.8-3.2 range gave vastly differing chromatograms in which WR 2721 was not separated from large interferences. A series of mobile phases containing different alkylammonium phosphates at pH = 2.8 produced some chromatograms in which WR 2721 was completely separated from all interferences and which gave selectivity in the order of peak elution. The concentration of ion-pairing reagent was 0.01M and CH^3CN/water ratio was approximately 1:4 in all systems tested. Each mobile phase was prepared by first titrating 0.1 moles of the amine in 70 mL water to pH 2.5 with conc. H_3OPO_4 and diluting to 100 mL, then mixing 1 part of the concentrate with 77 parts of water and 22 parts of CH_3CN.

Three systems gave the required separation - those using dicyclohexyl-, di-n-hexyl- and tetrabutylammonium phosphate (DCHAP, DHAP and TBAP). Figures 14-19 are HPLC traces of spiked and unspiked plasma made under each of the three conditions. The system containing dicyclohexylammonium phosphate caused WR 2721 to elute between two small endogenous peaks while the other two systems gave virtually identical chromatograms, with WR 2721 eluting <u>after</u> the two small peaks.

The tetrabutylammonium phosphate mobile phase was determined to be preferable because of slightly better resolution relative to dicyclohexylammonium phosphate, and was easier to prepare relative to di-n-hexylammonium phosphate.

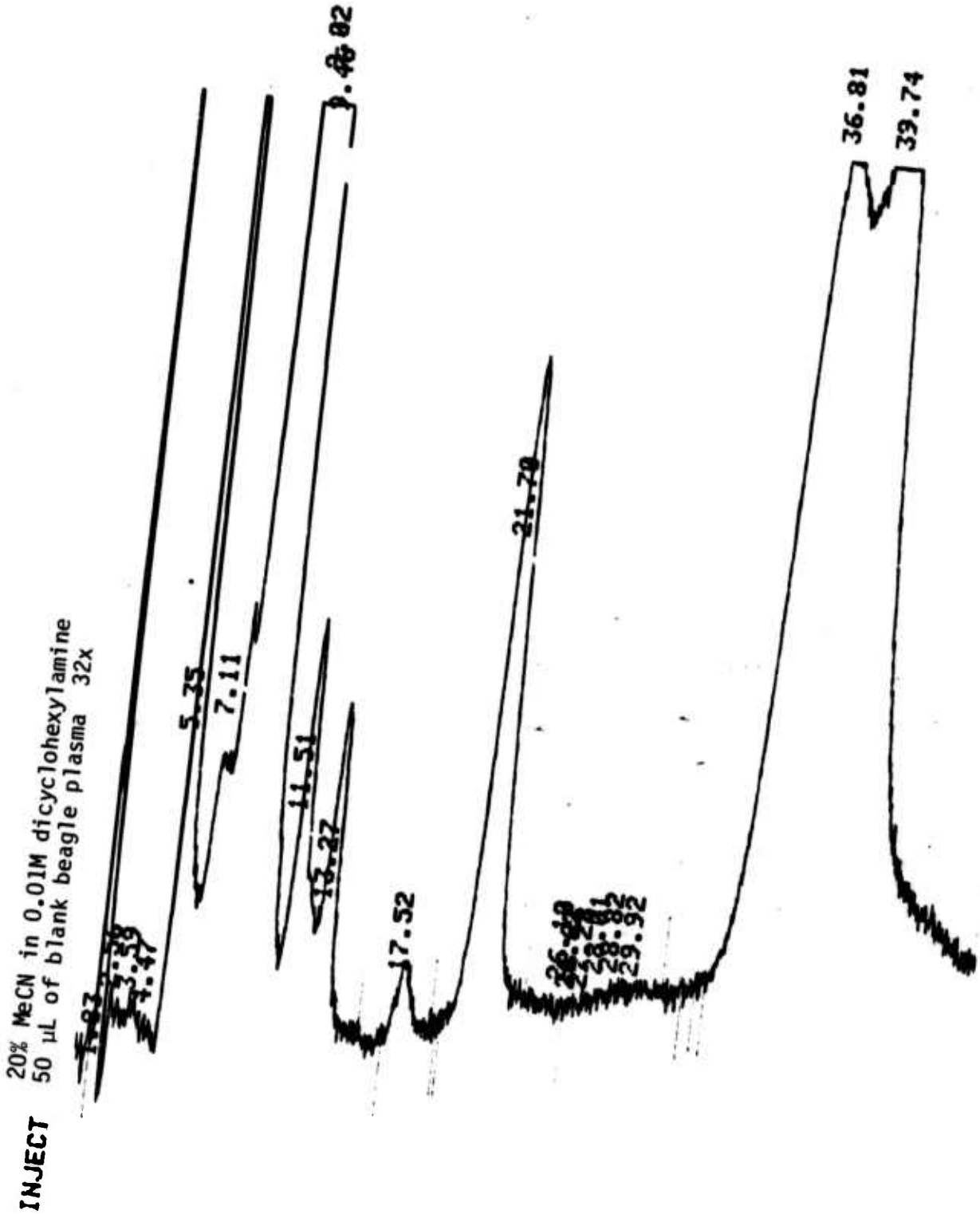


Figure 14. HPLC Analysis of Beagle Plasma Prior to Dosing. Mobile Phase 0.01M in Dicyclohexylammonium Phosphate.

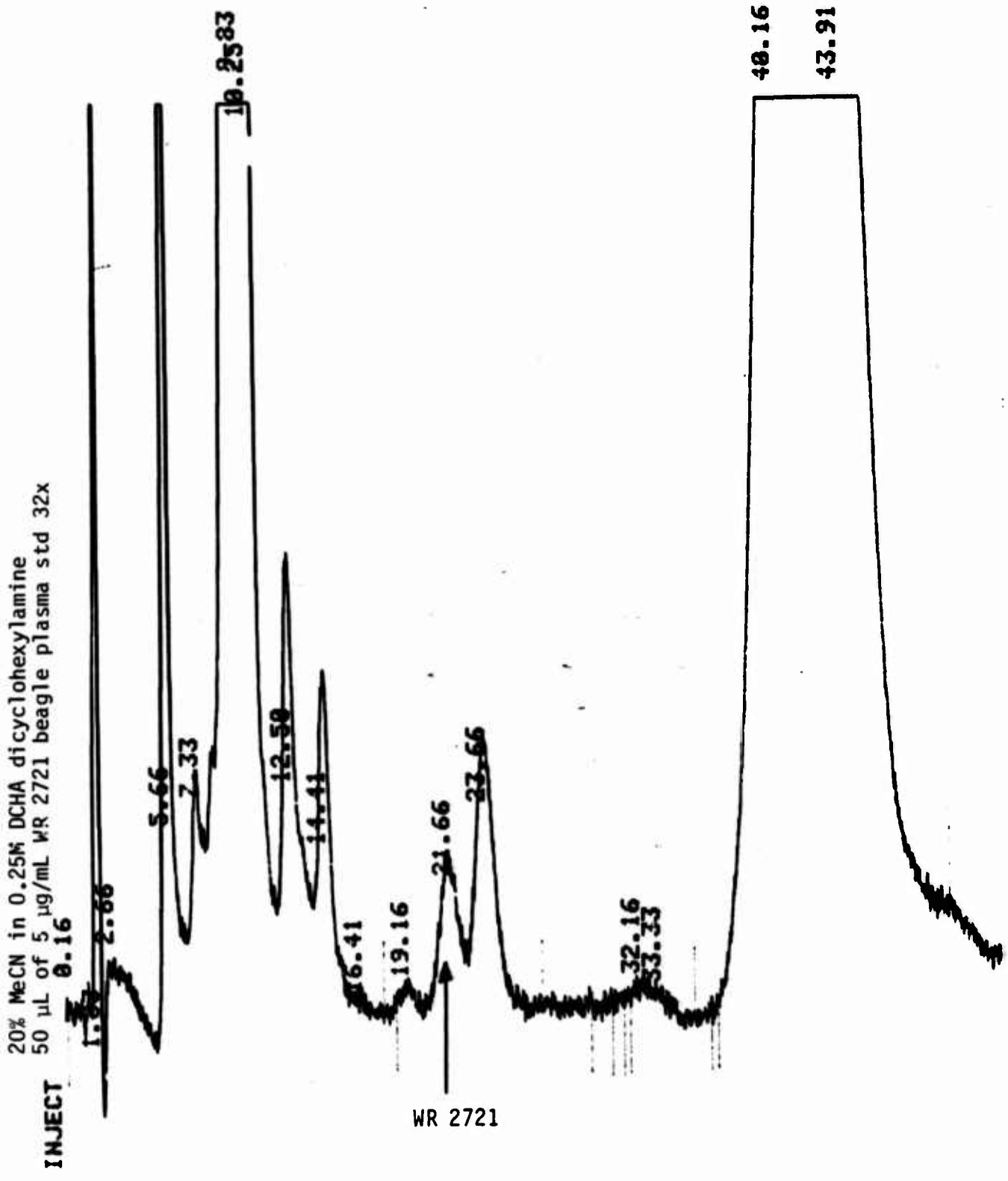


Figure 15. HPLC of Beagle Plasma Spiked with WR 2721 (5 µg/mL). Mobile Phase 0.01M in Dicyclohexylammonium Phosphate.

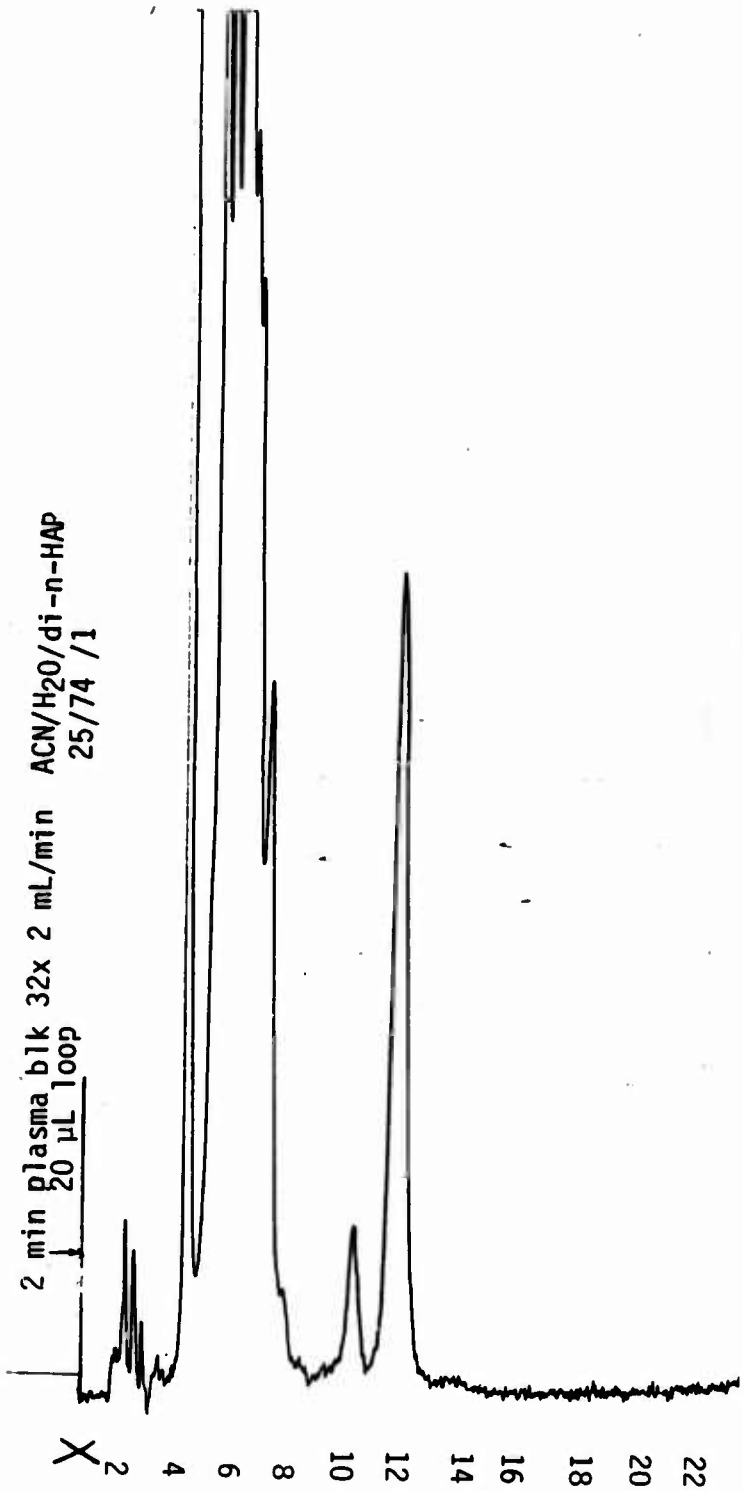


Figure 16. HPLC Analysis of Beagle Plasma Prior to Dosing Mobile Phase 0.001M in Di-n-hexylammonium Phosphate.

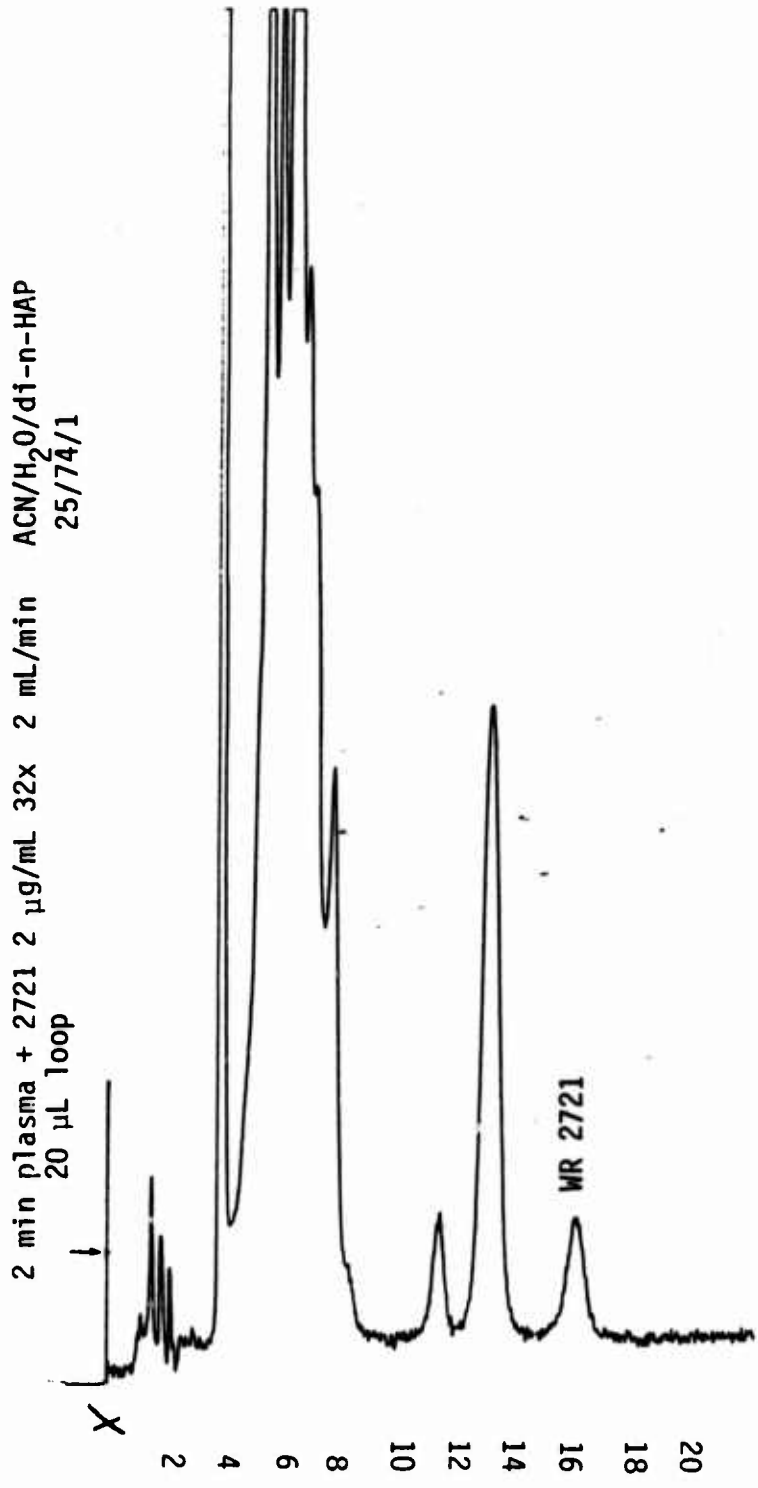


Figure 17. HPLC Analysis of Beagle Plasma Spiked with WR 2721 (2 µg/mL). Mobile Phase 0.001M in Di-n-hexylammonium Phosphate.

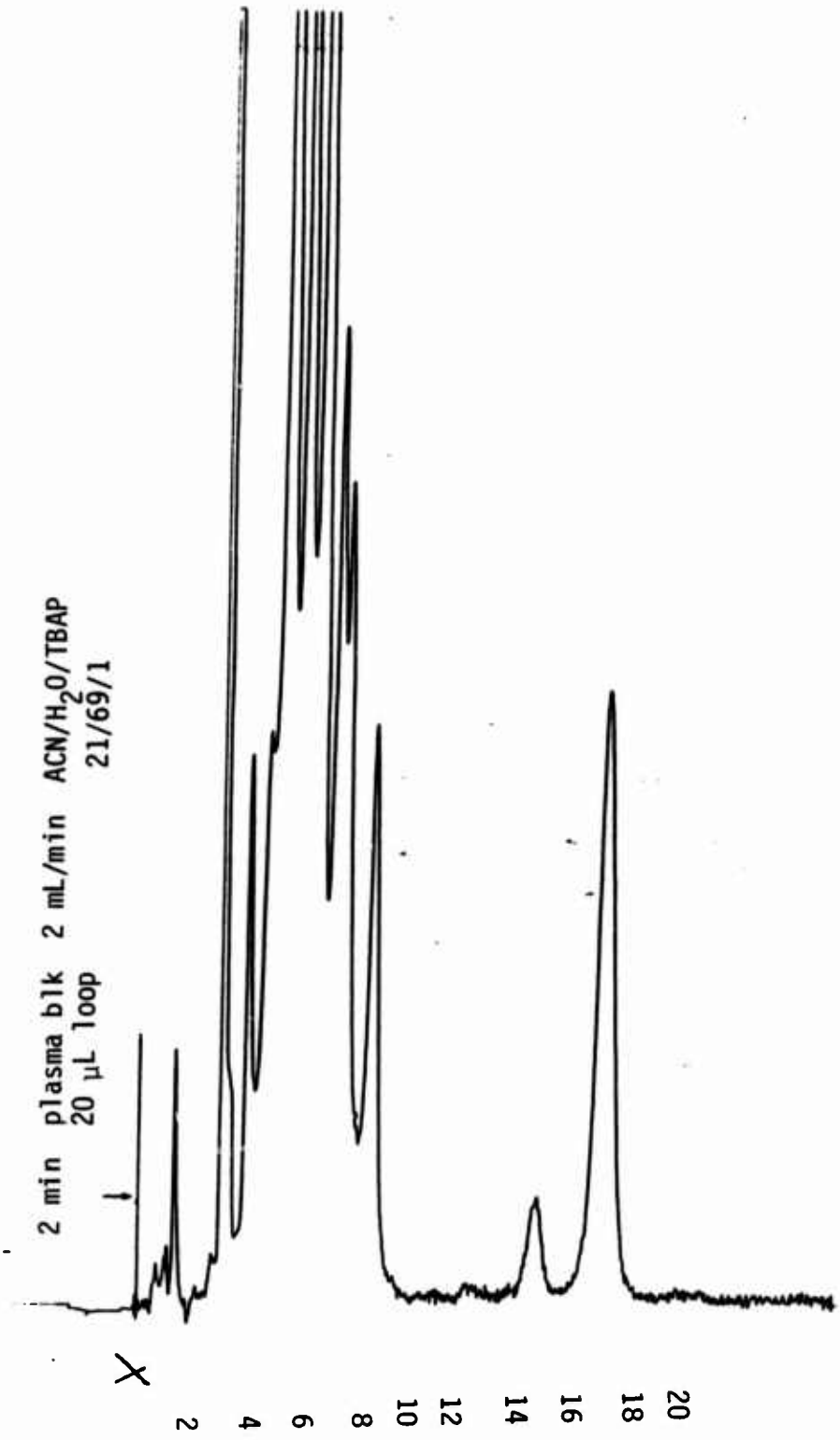


Figure 18. HPLC Analysis of Beagle Plasma Prior to Dosing. Mobile Phase 0.01M in Tetrabutylammonium Phosphate.

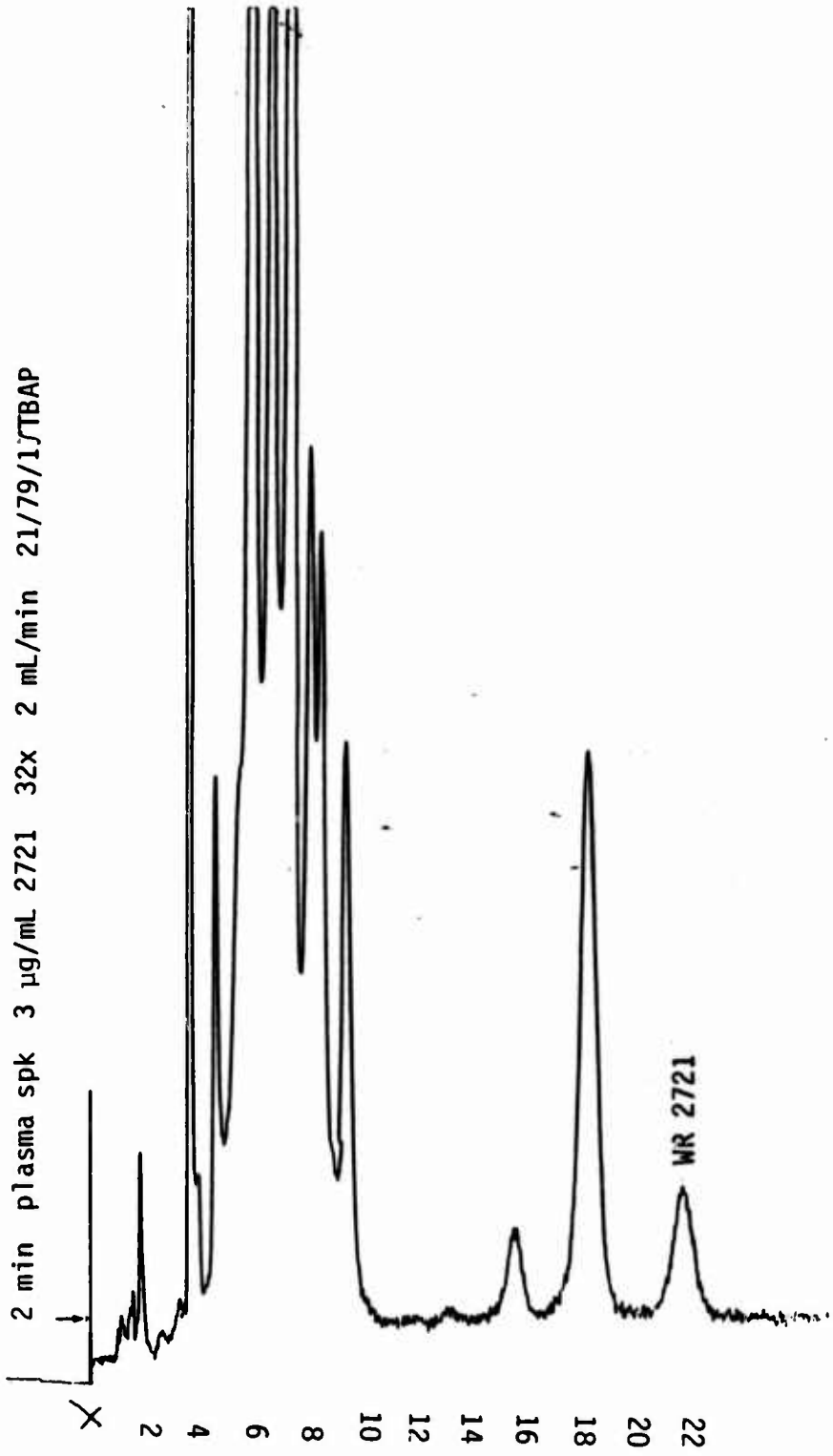


Figure 19. HPLC Analysis of Beagle Plasma Spiked with WR 2721 (3 µg/mL). Mobile Phase 0.001M in Tetrabutylammonium Phosphate.

A mobile phase of CH_3CN/water (22:78) 0.01M in TBAP, pH 2.8 was used to analyze beagle plasma taken from the test animal prior to and two hours after feeding. Three small peaks appearing immediately before the elution time of WR 2721 increased by factors of 7.9, 3.4 and 2.3 in order of emergence (Figures 20 and 21). No peaks were observed which would interfere with WR 2721 (Figure 22).

A sample of plasma from the same dog was spiked with 100 µg/mL of WR 2721 and 100 µg/mL of WR 80855 [S-3-(4-aminobutylamino)propylphosphorothioate] and analyzed with the TBAP system. The HPLC trace (shown in Figure 23) demonstrates that the homologs chromatograph well and are nearly baseline-separated. This experiment implies that an internal standard other than ^{14}C-labeled WR 2721 may be used with the newly-developed separation system.

3. Stability of WR 2721

a. In Plasma at 23°C

It was observed that when WR 2721 was added to beagle plasma and the mixture was allowed to stand a short time before derivatization and HPLC analysis, considerable variations in quantitation were observed. An experiment was carried out in which an interval was inserted between time of addition of WR 2721 to plasma (to give 80 µg/mL) and time of addition of derivatizing reagent, the mixture being allowed to stand at room temperature (23°C) during the interval. The amount of WR 2721 detected declined over 30 minutes to 78% of the original assay (Figure 24). This lack of stability has serious implications, for development of analytical protocol for the planned animal studies must take it into account.

b. In Plasma at -20°C

The stability of WR 2721 in beagle plasma stored at -20°C was determined. Plasma was spiked with WR 2721, and the mixture was divided into 100-µL aliquots which were quick-frozen in a Dry Ice/acetone bath. These samples were stored in a freezer at -20°C and periodically removed for analysis. The peak area for WR 2721 was normalized using each of two peaks from endogenous plasma components (elution times of 22 and 27 minutes) as ''internal standards.'' WR 2721 concentration was essentially unchanged over a 28-day period. Setting the zero-time analysis at 1.00, the average of five analyses (0, 1, 2, 17, and 28 days), was 1.05 using Peak 1 and 1.08 for Peak 2. Standard deviations were 0.13 and 0.12, respectively. Normalized peak areas are presented in Table 7 and representative chromatograms are given in Figure 25.

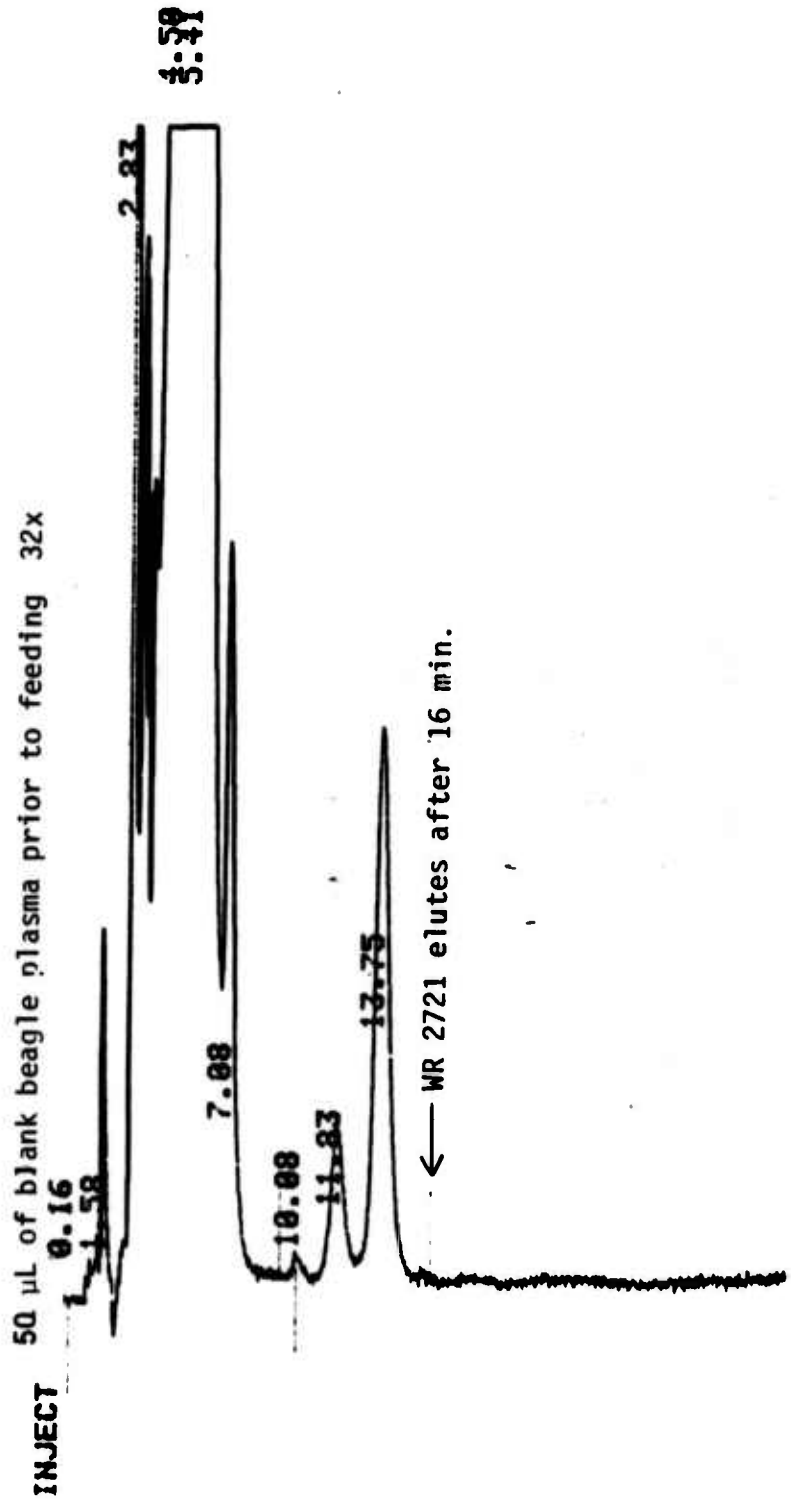


Figure 20. HPLC Analysis of Beagle Plasma Taken from Animal Immediately Prior to Feeding.

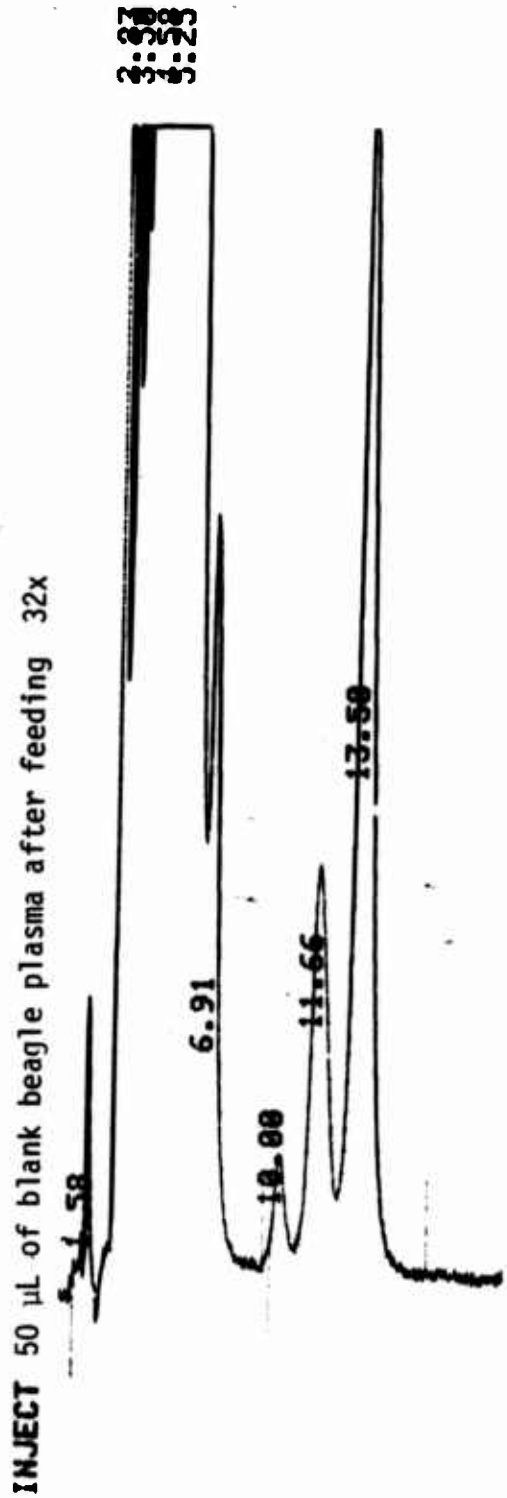


Figure 21. HPLC Analysis of Beagle Plasma Taken from Animal Two Hours After Feeding.

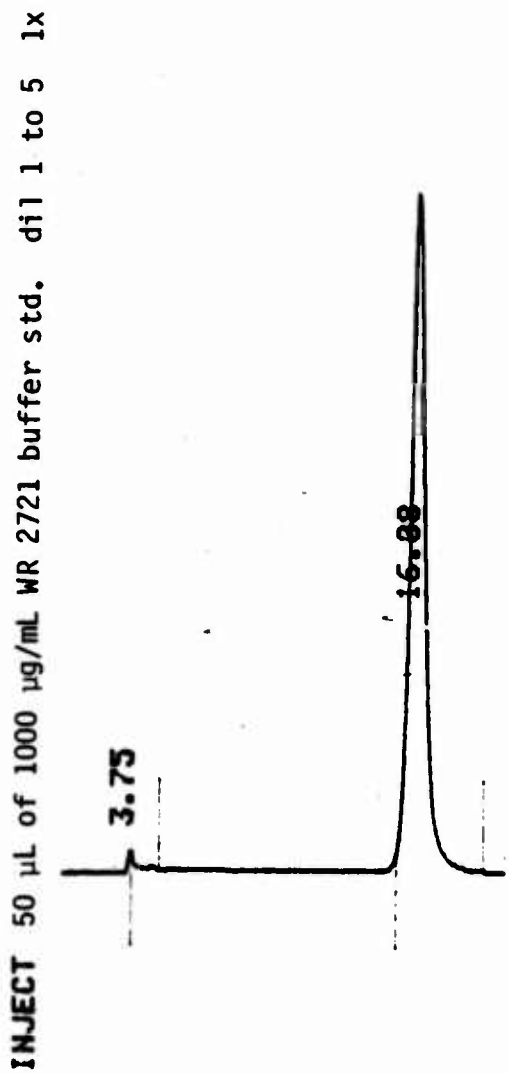


Figure 22. HPLC Analysis of WR 2721 in Buffer Solution Showing Its Retention Time Under Same Conditions Used to Produce Figures 20 and 21.

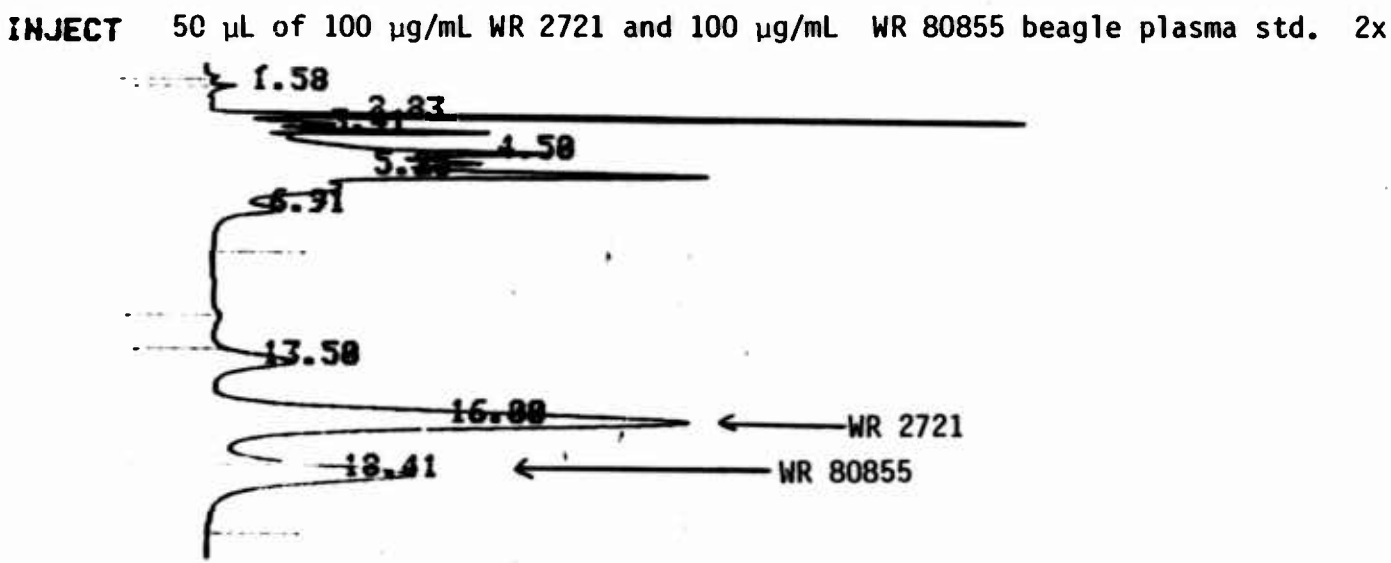
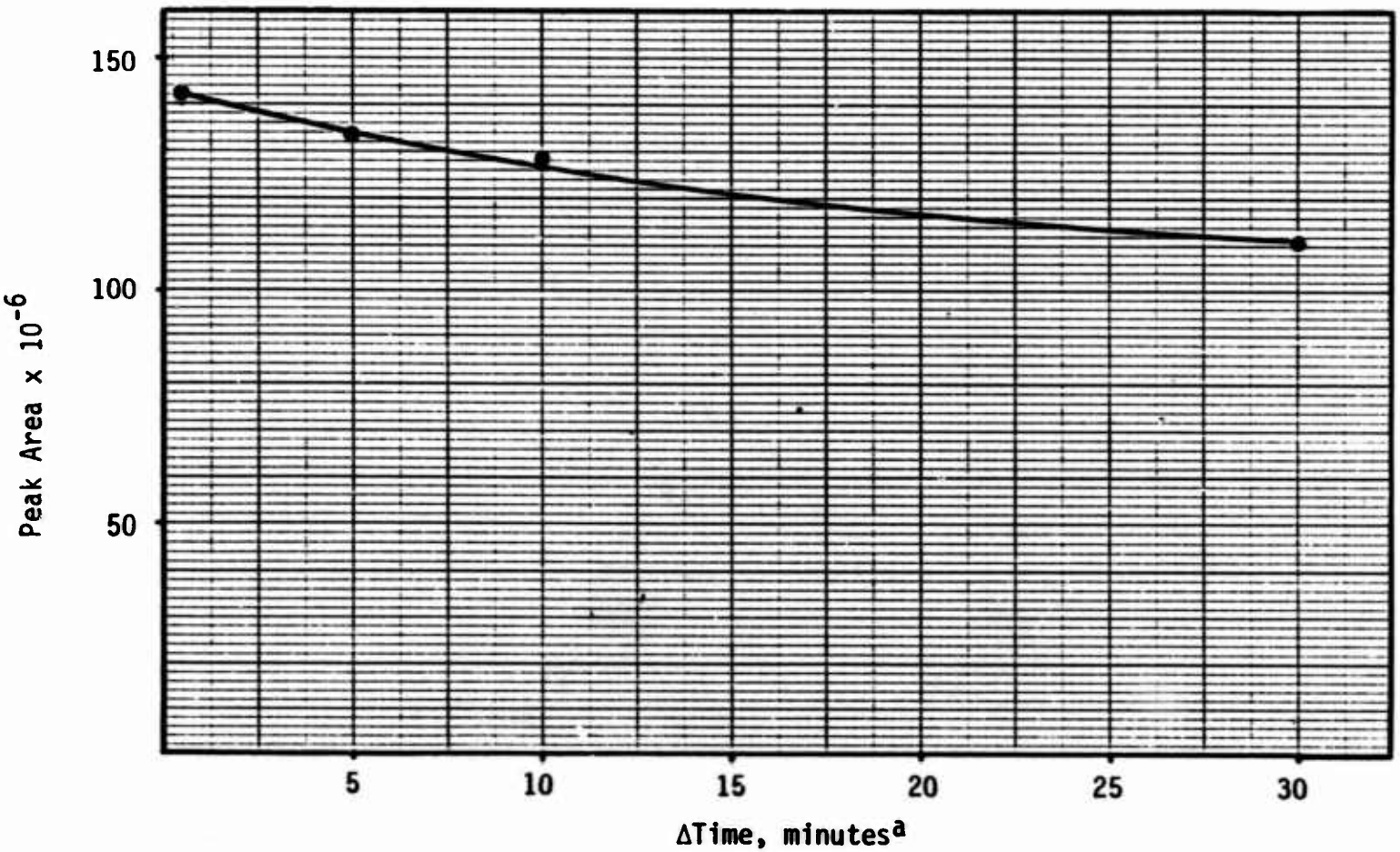


Figure 23. HPLC Analysis of Beagle Plasma Spiked with WR 2721 and WR 80855.



a. ΔTime - time interval between addition of WR 2721 to beagle plasma (80 µg/ml) and addition of fluorescamine.

Figure 24. Analysis of WR 2721 in Plasma at 23°C.

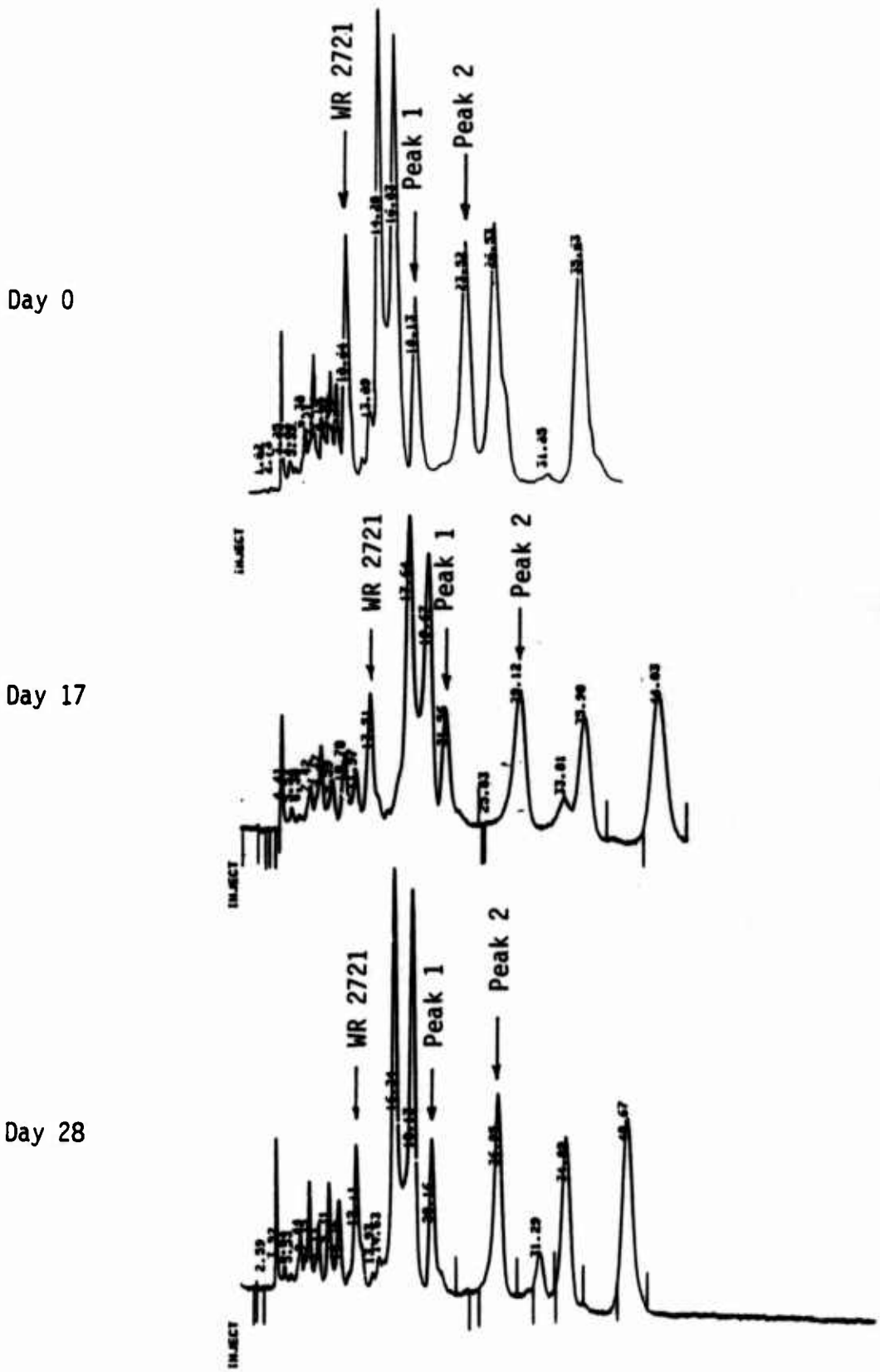


Figure 25. Chromatographs from HPLC Analysis of WR 2721 in Beagle Plasma Stored at -20°C.

TABLE 7. HPLC ANALYSIS OF WR 2721 IN FROZEN BEAGLE PLASMA

Day	Normalized Peak Area By Peak 1	By Peak 2
0	1.00	1.00
1	0.84	0.90
2	1.16	1.12
17	1.15	1.21
28	1.08	1.15
	$\bar{x}$ = 1.05	$\bar{x}$ = 1.08
	s = 0.13	s = 0.12

c. Stability of Fluorescamine Derivative of WR 2721

Stability of the fluorescamine derivative of WR 2721 was determined. A buffer solution (pH 7.6) containing 80-μg WR 2721 per milliliter was derivatized and analyzed repeatedly by automatic injection. Area of the WR 2721 peak declined steadily over three hours. A plot of log[area] versus time (Figure 26) was linear with a correlation of 0.9995. Assuming the decomposition to follow pseudo-first order kinetics, the half-life of the derivative was 51 minutes. Also tested was WR 159023, a homolog of WR 2721. Its fluorescamine derivative decomposed at a similar rate, with a half-life of 46 minutes. The mechanism of the decomposition is not known.

4. Development of Other Analytical Methods for WR 1065 in Plasma

a. Derivatization with Fluorescamine

Concurrent with the WR 1065 fluorescamine derivatization studies under Section IV the feasibility of extracting the WR 1065 fluorescamine derivative from plasma was also investigated. Previous attempts to extract the WR 2721 fluorescamine derivative from plasma had shown that extraction with less polar and hydrophobic solvent systems had resulted in a preferential concentration of the WR 1065 metabolite form over the WR 2721. Accordingly, these types of solvent systems were investigated for the extraction of WR 1065; examples of these systems are listed in Table 8.

TABLE 8. SOLVENT SYSTEMS EVALUATED FOR EXTRACTION OF WR 1065-FLUORESCAMINE DERIVATIVE FROM AQUEOUS PHASES

ethyl acetate	toluene
acetone-DCM	DCM
acetone-toluene	acetone-ethyl acetate-DCM
acetone-ethyl acetate-toluene	acetone-DCM-heptane
acetone-pet ether-DCM	

DCM - dichloromethane (methylene chloride)
For solvent mixtures, a wide range of solvent compositions were compared.

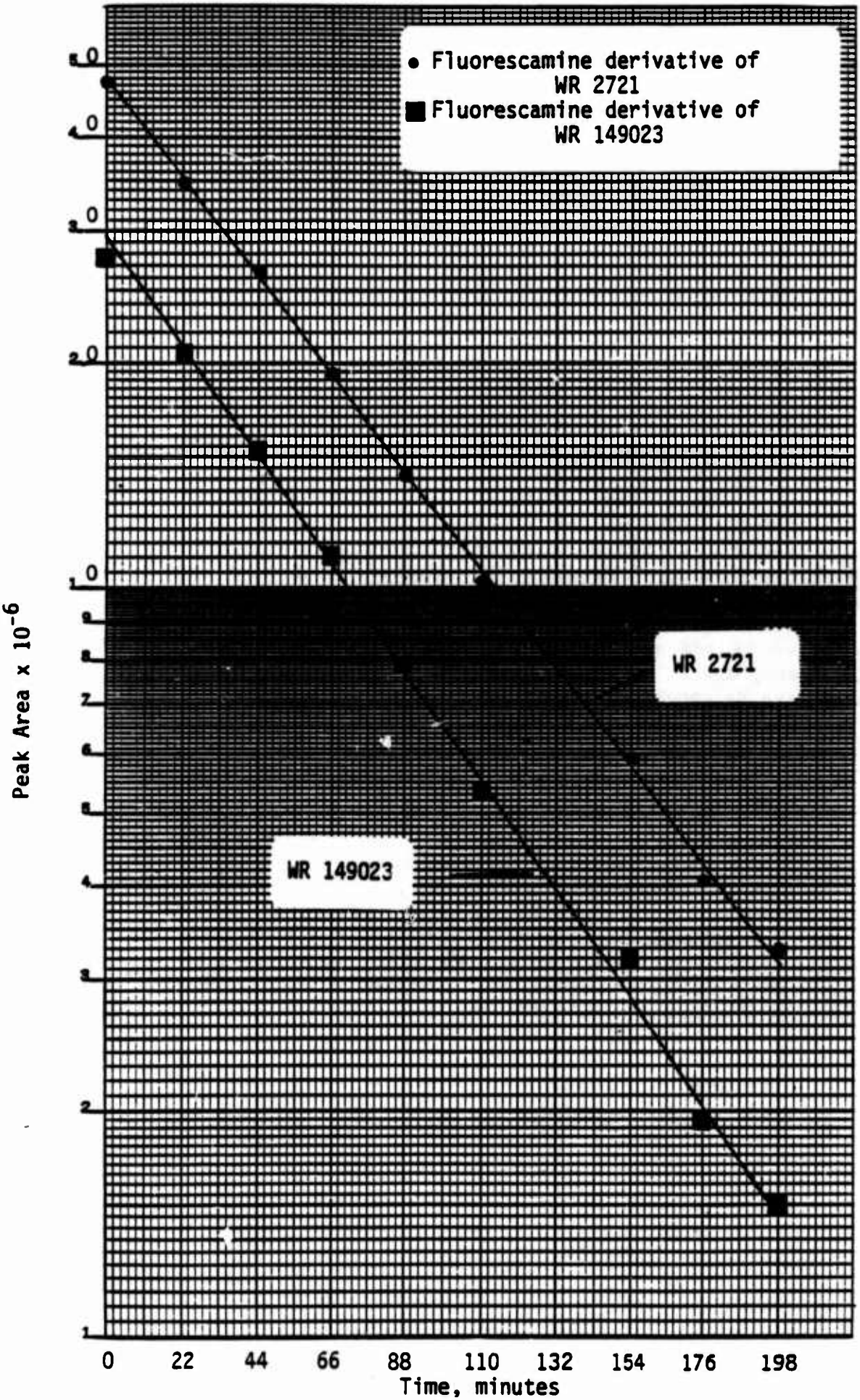


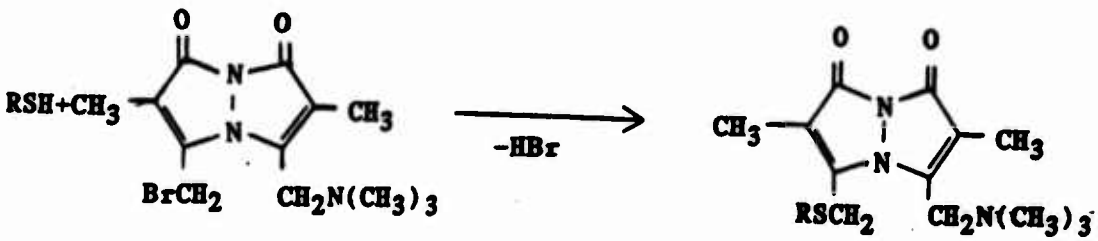
Figure 26. Decomposition of Fluorescamine Derivatives at 23°C.

Solutions of the individual fluorescamine derivatives of WR 2721 and WR 1065 in both buffer and plasma were extracted with two sequential portions of the extracting solvent, and the fluorescences of both the starting solution and each extract were quantitated. While there were consistently significant differences in the amount of fluorescence recovered in the extracts using different solvent systems, none of the solvent systems were capable of quantitative recovery of WR 1065 or of preferential extraction of WR 1065 over WR 2721. It was necessary to include a water-miscible solvent for the fluorescamine moiety, such as acetone or dioxane, in order to extract much of either derivative into an immiscible phase and this, of course, resulted in the coextraction of both derivatives. It also apparently resulted in the inclusion of appreciable quantities of water in the organic phase causing more of the more polar WR 2721 form to be extracted than would be expected.

The above-described difficulties in the extraction of the fluorescamine derivatives appeared to be inherent limitations in the structure of the fluorescamine moiety due to the polar hydrophilic groups present on the fluorescamine ring structure. These influences apparently override the structural differences in polarity between the phosphate group and the sulfhydryl group on the other ends of the drug and its metabolite, making it very difficult to separate the two forms.

b. Derivatization with Bromobibane

An alternative approach, the derivatization of WR 1065 with bromobibane fluorescent labels as described by Fahey et al.[4,5] was considered. The use of bromobibane fluorescent labeling for biological thiols in general[5] and for WR 1065 in particular[4] have been described. The derivatization reaction is shown below.



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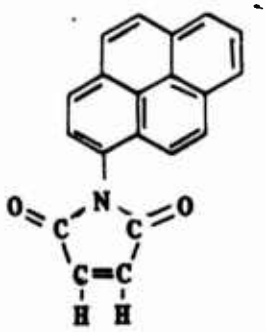
When applied to the analysis of WR 1065 in animal tissues, the recovery of WR 1065 from tissue was generally not satisfactory. These authors accordingly investigated the possibility of extraction of the WR 1065-bromobibane derivative; it was reported that a satisfactory extraction process had not yet been developed.[4] This may be due to the same reasons that make extraction of the fluorescamine derivative difficult, i.e., the presence of polar groups on the derivatizing molecule which interfere with the partitioning of the molecule from an aqueous phase into an immiscible organic phase.

c. Other Potential Derivatives

For the assay of a sulfhydryl-containing molecule such as WR 1065, there would appear to be important advantages to derivatizing the free sulfhydryl group instead of the primary amino group as with fluorescamine. Derivatization of this group might inhibit oxidation of the RSH group preventing formation of RSSR derivatives during analysis. A number of thiol derivatives described in the literature were considered for adaptability to the present application. Many of the classical thiol derivatization reactions such as the reactions with DTNB, DTT, DNFB, mercurials and other metals, and nonmetallic chelating agents, such as phenanthroline, are based on the formation of mixed disulfides with thiol of interest. Such exchange reactions can be reversed by the presence of free thiol or other reducing agents, and this reversibility complicates the recovery and quantitation steps in assay methodology. It would be preferable to derivatize RSH with a molecule which binds covalently and irreversibly with the free sulfhydryl group. One such derivatizing agent is described below.

d. Derivatization with N-(3-Pyrene)maleimide

Of the many types of molecules capable of reacting with sulfhydryl groups, one of the most specific types of reaction involves that of activated double bonds as present in N-ethylmaleimide (NEM) and acrylonitrile. Weltman et al.[6] described the synthesis and application of a sulfhydryl reagent containing both a maleimide moiety and a pyrene ring structure. The structure of this reagent, N-(3-pyrene)maleimide, is shown below.



The maleimide group binds to free sulfhydryl groups of thiols and after binding, the pyrene moiety becomes fluorescent thus acting as a covalently bound fluorescent label. Of particular interest to the present application, the pyrene ring structure is also markedly hydrophobic and this property might significantly aid in the extraction and separation of the WR 1065 derivative.

In preliminary experiments, the use of N-(3-pyrene)maleimide to form a fluorescent derivative of RSH was explored. N-(3-pyrene)maleimide dissolved in a mixture of ethanol, acetone and toluene was added to a solution of WR 1065 in Clark and Lubs borate buffer (pH 7.7) containing 10% (v/v) acetone plus a small amount of potassium borohydride as reducing agent. The reaction mixture was allowed to sit (with periodic vortexing) for 15 to 20 min and then

observed for fluorescence. The fluorescent spectrum obtained is shown in Figure 27. The reaction appears to be complete within a few minutes. The fluorescent derivative formed also appears to extract easily into a hydrophobic organic phase of toluene-heptane, although no attempt at quantitation was made in these experiments. On the basis of these preliminary experiments, this fluorescent derivative of WR 1065 appears very promising for use in WR 1065 analysis.

5. Simultaneous HPLC Analysis of WR 2721 and WR 1065 in Beagle Plasma

Although development of a gradient system for the simultaneous HPLC analysis of WR 2721 and WR 1065 was not a stated objective of the current work, it was convenient to carry out a preliminary experiment to determine the feasibility of such an analysis. Using a buffer solution of both compounds at the 80 μg/mL level, aliquots were derivatized in the usual way and subjected to HPLC analysis in which the acetonitrile concentration was continuously increased during development of the chromatogram. Under the following conditions, WR 2721 was completely separated from all other peaks and WR 1065 had only a minor interference which was partially separated. (See Figure 28).

Column:	RCM-100 10 μm C-18
Mobile Phase:	Binary gradient, 75% A to 65% B, Profile 8
Solvent A:	0.01M dibutylammonium phosphate, pH 2.8
Solvent B:	acetonitrile/water, 900, 0.01M in dibutylammonium phosphate, pH 2.8

It is anticipated that this separation could be improved without extending its current total analysis time of <60 min.

B. In Vivo Studies

1. Administration of WR 2721 to the Animal

A solution of WR 2721 in isotonic saline (125 mg/mL anhydrous concentration) was administered via an indwelling catheter in one of the cephalic veins over a two minute period. The total delivered dose was determined by the animal weight, as the drug is given at the rate of 150 mg/kg body weight. After drug administration, the catheter was flushed with 3-5 mL of normal saline. (More detailed protocols are included in Appendix A.)

2. Collection, Fractionation and Preservation of Blood Samples

Blood samples were withdrawn via a cannula placed in the opposite jugular vein and were collected in EDTA vacutainers. Samples (10 mL) were collected prior to dose administration and at the nominal times of 1, 2, 3, 6, 9, 12, 15, 22, 30, 45, 60, 90, 120, 150, 180, 210, 240, 300 and 360 minutes post infusion (3.0 mL). Because the drug administration was over a two minute period, time

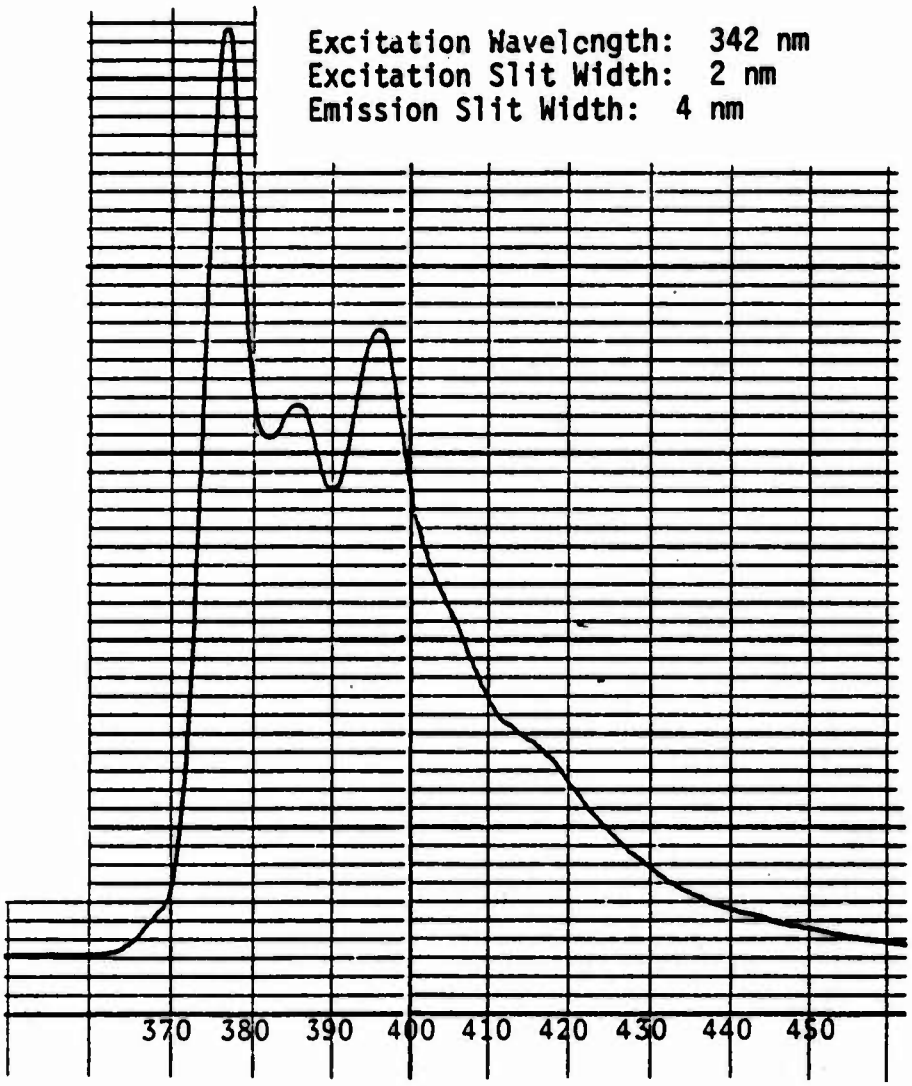


Figure 27. Emission Spectrum of WR 1065/N-(3-Pyrene)maleimide Adduct in Clark and Lubs Borate Buffer, pH 7.7, 10% (v/v) Acetone; KBH_4 Added as Reducing Agent.

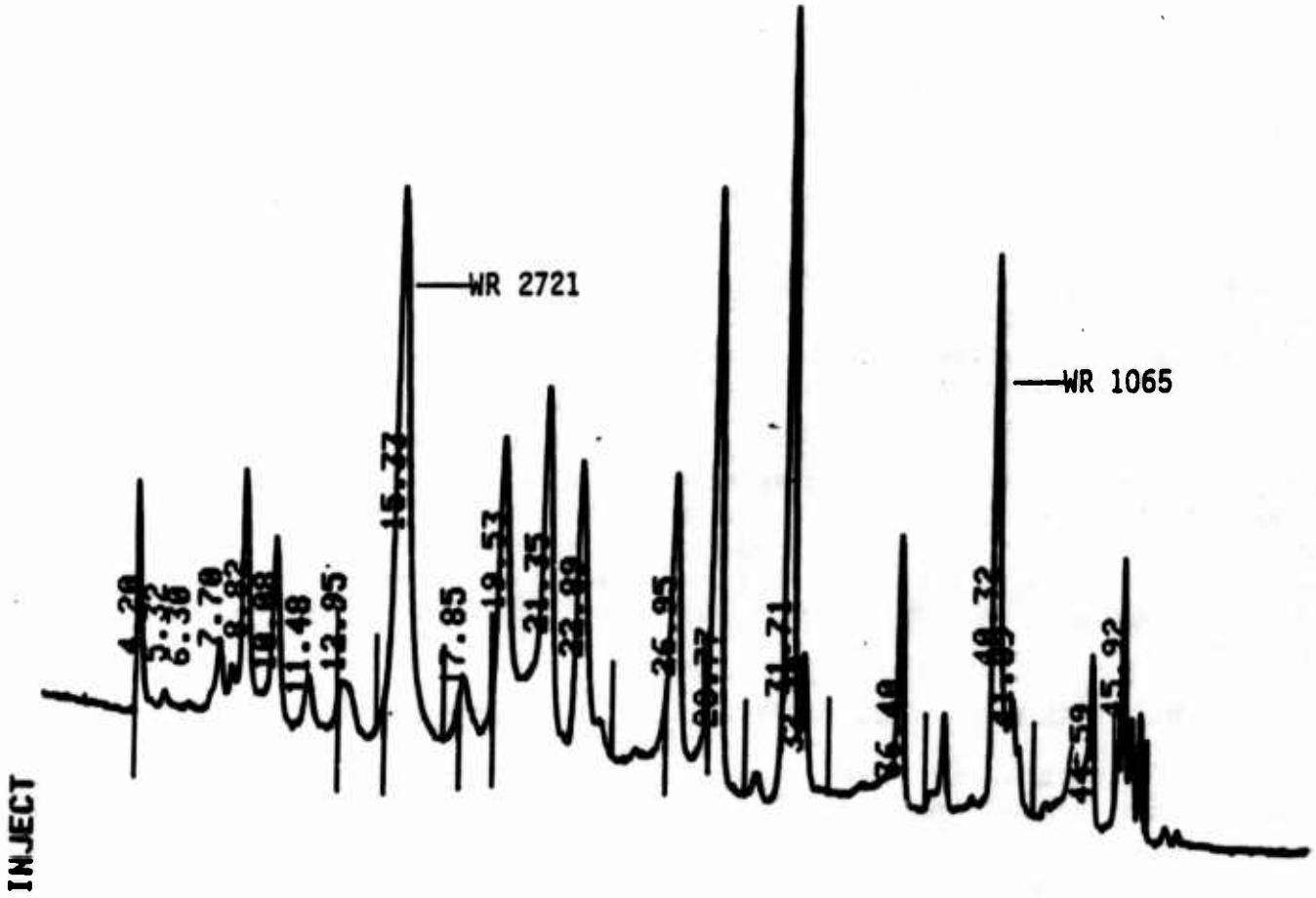


Figure 28. Gradient HPLC Analysis of WR 2721 (80 µg/mL) and WR 1065 (~50 µg/mL) in Beagle Plasma.

zero was set at the end of the infusion period. The samples were immediately chilled in ice water and centrifuged at 2000 rpm for 10 minutes. Ninety (90) µL portions of plasma were removed, placed in plastic assay tubes, and frozen at -70°C by immersing the outside of the tube in a Dry Ice/isopropanol bath. The remainder of the blood and plasma samples were also frozen at -70°C and stored at -20°C.

3. Addition of Internal Standard

S-[2-(3-aminopropylamino)ethyl-1,2-^{14}C] phosphorothioic acid (WR 2721) specific activity 86.0 µCi/mg was used as an internal standard. Seventy (70) ng of ^{14}C-labeled WR 2721 (16,700 dpm) was added to each 90-µL portion of beagle plasma prior to freezing. This is equivalent to a plasma background WR 2721 level of 0.776 µg/mL. Radiolabeled WR 2721 was chosen because it will undergo the same chemical reaction at virtually the same rate as its unlabeled congener.

Thus, by determining the amount of radioactive WR 2721 present in the assay relative to the amount added at the time the plasma was frozen, the analyst can calculate for each assay the percent recovery of the administered drug.

a. Preparation of Standards

Unlabeled WR 2721 was dissolved in 0.1M Clark and Lubs pH 10 borate buffer to give a solution having a final concentration of approximately 1 mg/mL anhydrous WR 2721. One-mL portions of this solution were placed in separate screw-capped tubes, frozen and lyophilized. The lyophilized material was stored at -20°C, and rehydrated immediately before use.

^{14}C-labeled WR 2721 was likewise dissolved in 0.1M Clark and Lubs pH 10 borate buffer to give a solution having a concentration of 1.4 ng/µL ^{14}C-WR 2721 (334 dpm/µL). One-mL portions of this solution are frozen, lyophillized and stored frozen at -20°C. On each day samples were analyzed a 1.0-mL portion was rehydrated and used for internal standard addition. The solution after rehydration was kept at 0 to 4°C and any remaining after the day's analyses was discarded.

b. Determination of Recovery of WR 2721, Sample Calculations

Each assay tube containing 90 µL of beagle plasma and 50 µL of internal standard solution was thawed immediately prior to analysis, derivatized with fluorescamine and adjusted to a final volume of 700 µL as shown in Table 9.

TABLE 9. COMPOSITION OF PLASMA ASSAYS SOLUTION

Reagent	µL/Assay Tube	Cumulative Volume, (µL)	^{14}C-WR 2721 added, dpm
Plasma + Internal Standard	140	140	16,700
Fluorescamine in Acetone (5 mg/mL)	2 X 200	540	0
Clark and Lubs pH 7.6 buffer	160	700	0

A 50-µL portion of this assay mixture was injected via a stainless steel sampling loop onto the HPLC column for analysis. This corresponds to injection of 1190 dpm of ^{14}C-WR 2721 if all is recovered. One-mL volumes of column eluant were collected in mini-scintillation vials, diluted with 4.0 mL of scintillation fluid and counted for 10.0 minutes using a Packard Model TriCarb scintillation counter. A vial containing only mobile phase and scintillation fluid was also counted. This background value (approximately 30 dpm) was then subtracted from all experimental samples prior to summation of the values for those vials exhibiting greater than background radioactivity.

A 50-µL portion of each derivatized assay mixture was also added to 1.0 mL of mobile phase, diluted with scintillation fluid and counted directly to ascertain the actual amount of radioactivity present in 50 µL of the assay mixture.

On each day samples were processed a standard 89 µg/mL of WR 2721 buffer solution containing 0.776 µg/mL ^{14}C-WR 2721 was analyzed. Analysis of this sample allowed day-to-day instrument response to be monitored and the day's results to be adjusted accordingly. Sample data are shown in Table 8.

Adjusted peak areas were calculated using the following formula:

$$AREA_{adj} = \frac{AREA \times DPM \text{ added}}{GAIN \times DPM \text{ recovered}}$$

where

AREA = raw integrator area in intergrator units, or peak height in arbitrary units (usually mm.)

GAIN = fluorescence detector instrument gain

DPM = background corrected radioactivity (in dpm) found in 50 µL portion of assay mixture

DPM recovered = background corrected radioactivity found in WR 2721 peak from HPLC analysis of 50 µL portion of sample assay mixture

As demonstrated by the adjusted peak area values in Table 10, the instrument may exhibit time-related changes in sensitivity. The analysis of the 89.7 µg/mL buffer standard on a daily basis allowed each days' experimentally determined values to be adjusted to a common basis.

TABLE 10. SAMPLE AREA AND INTERNAL STANDARD ACTIVITY DATA

Date of Analysis	Sample	Peak Area Arbitrary Units	dpm 50 µL Added	dpm 50 µL Recovered	Instrument Gain and Recovery Adjusted Peak Area
12-21-82	89.7	132645	1254	666	62439
12-22-82	89.7	124847	1212	584	64782
12-01-82	89.7	129942	1276	460	90112
12-06-82	89.7	133916	1174	480	87733

4. Plasma Level Time Course

Table 11 and Figure 29 present the results obtained from three animal dosing experiments. These experiments were performed over a period of three months and allowed the compound administration, blood collection and analytical procedures to evolve to their present level.

TABLE 11. RESULTS FROM PRELIMINARY ANIMAL DOSING STUDIES

Post Infusion Sample Collection Time (minutes)	Plasma WR 2721 Concentration (µL/mL) 10-04-82	10-13-82	12-06-82
1		1030	925
2		888	583
3	1840	789	436
6		560	420
9		371	266
12		251	368
15	789	195	254
22		147	99
30	121	83	63
45		30	26
60		17	3
90		8	2.8
120	27	7	1.7
150		11	2.1
180		8	2.4
210		9	2.8
240		10	3.5
300		12	3.9
360		8	4.3

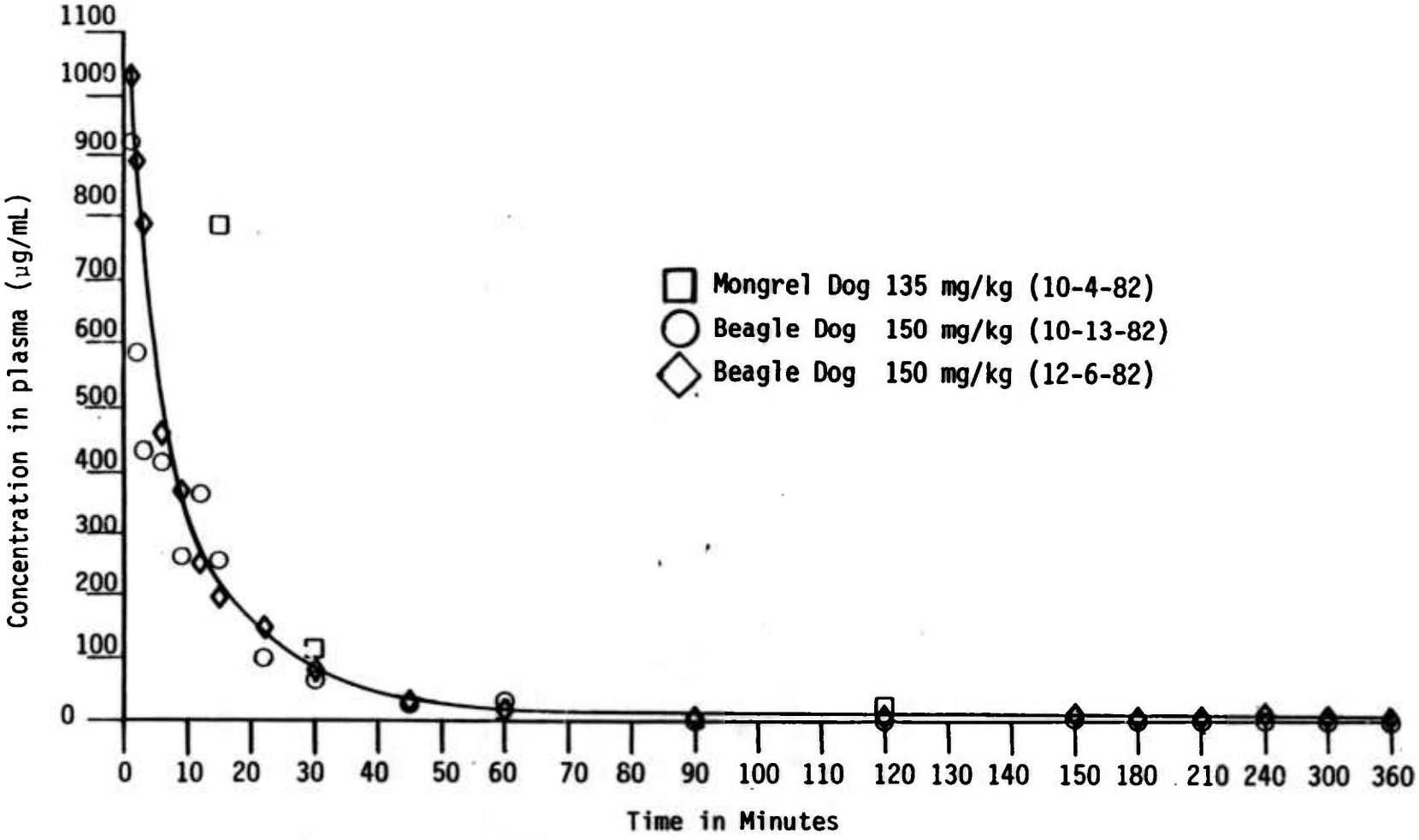


Figure 29. Observed WR 2721 Dog Plasma Concentrations as a Function of Post Infusion Time.

C. Microencapsulation

A series of encapsulation runs was conducted to determine the highest payload of WR2721 which could be incorporated into microspheres and microcapsules. For these runs, a standard particle size of WR 2721 (<105 microns) was used. Conditions for the encapsulation runs are presented in Tables 12 and 13. Ambient temperature was held constant at 22.2°C. Microspheres up to 40 percent payload were successfully prepared with about 35 percent appearing to be the optimal loading. Microcapsuleswith up to 35 percent loading of the fill (23 percent overall payload) were also successfully prepared. Runs 1-56 through 1-63 were conducted to prepare microspheres containing varying amounts of stearic acid as the matrix with and without triglyceride at a 35 percent loading. In all cases the capsules made well but evaluation of these samples was postponed pending development of the plasma assay.

1. Hydrolytic Stability

Hydrolytic stability tests at pH 1 were carried out on samples from 26 experimental encapsulation runs. A ''protection factor'' has been defined as the fraction of WR 2721 remaining in the formulation after testing divided by the fraction of unencapsulated WR 2721 remaining after being subjected to the same acidic conditions. This factor has been calculated for all formulations and the data are presented in Table 14. Seven runs produced samples which retained greater than 67 percent of the original payload at the conclusion of the test, with protection factors ranging from 6.7 to 9.4. One of these samples (1-53B) was clearly superior, with 94.7 percent of the drug being recovered. Microsphere sample 1-44, having a 40 percent payload in a matrix of 50/50 by weight stearic acid/triglyceride, showed poor protection of WR 2721 with only 41.6 percent recovery. Microcapsules with a 35 percent payload (23 percent overall) gave good protection of WR 2721 with 82.6 to 85.6 percent recovery.

2. In Vitro Release Rates

In vitro release rate studies in intestinal fluid were conducted on three samples and the results are illustrated in Figures 30 and 31. Near complete release of WR 2721 occurred within 1.0 hour with possibly some slight decomposition of the WR 2721 after 1-2 hours.

3. Aging Stability

Preliminary thermal stability tests of four samples prepared previously were carried out at 37°C for one month. Results are presented in Table 15. Assay of the samples indicated 77.6 to 94.4 percent of the original WR 2721 was recovered.

The hydrolytic stability of the aged samples was also determined. Protection factors ranged from 2.6 to 7.4 (Table 15) with the stearic acid/triglyceride matrix being superior to stearic acid/paraffin.

In vitro release testing of a sample from Run 1-22 indicated complete release of the WR 2721 into intestinal fluid within one hour (Figure 32).

TABLE 12. MICROSPHERE PRODUCTION[a,b,c,d,e,]

Run No.	Matrix Composition (%)[f] WR 2721	Dynasan 114	Softisan 154	Grocol 600-E	Emersol 6349	Paraffin Wax 140/145°F	Atmul 84K	Temp. of System (°C)	Anal. % WR 2721	Size (u) Distribution (%) 250-500	500-710	<250+ <710	Comments
1-44	40.0			30.0	30.0			68.3	36.1	17.1	82.9		Made well.
1-45A	34.9				56.9	8.2		"	28.7	12.6	85.6	1.8	Made well.
1-45B	34.9				48.8	16.3		"	25.1	10.6	78.8	10.6	Made well.
1-45C	35.0				40.7	24.3		"	26.3	14.4	80.6	5.0	Made well.
1-46A	35.0			45.4	19.6			"	33.8	7.8	80.2	11.9	Made well.
1-46B	35.0			26.0	26.0	13.0		"	34.7	9.8	76.9	13.3	Made well.
1-46C	35.0			19.6	45.4			"	33.4	7.3	68.0	24.7	Made well.
1-47A	35.0				48.8	16.2		"	36.5	8.4	71.1	20.5	Made well. WR 2721 was dispersed in steeric acid first and paraffin wax added to determine if it would help in the wetting and dispersion of WR 2721.
1-48A	35.0			26.0	26.0		13.0	"	34.2	8.1	65.6	26.3	Made well.
1-48B	35.0				44.6	13.0	7.4	"	32.8	9.3	69.6	21.1	Made well.
1-49	35.0				52.0	13.0		"	33.0	17.2	54.3	28.5	Made well.
1-53A	40.0				48.0	12.0		"	33.2	8.8	73.7	17.5	Made well. Had some shell/-fill temperature problem.
1-53B	35.0			13.0	52.0			"	32.3	12.9	75.5	11.6	Made well.
1-54A	35.0				61.6	3.4		"	33.4	10.6	69.0	20.4	Made well. Had more collection lost than normal.
1-54B	30.0				66.6	3.4		"	29.9	13.3	69.4	17.3	Made well.
1-54C	30.0			21.0	49.0			"	28.1	16.2	78.7	5.1	Made well.
1-55	35.0		19.6		45.4			"	32.8	3.9	81.9	14.2	Made well.
1-56	35.0				65.0			"	-	14.5	85.5		Made well.
1-57	30.0				70.0			"	-	11.4	75.4	13.2	Made well.
1-58	35.0			6.5	58.5			"	-	9.9	76.6	13.5	Made well.
1-59	35.0		13.0		52.0			"	-	13.8	76.0	10.2	Made well.
1-60	35.0		6.5		58.5			"	-	5.1	81.2	13.7	Made well.
1-61	35.0				65.0			"	-	11.3	79.7	9.0	Made well.
1-62	35.0			13.0	52.0			"	-	8.0	80.0	12.0	Made well.
1-63	35.0	13.0			52.0			"	-	9.9	77.1	13.0	Made well.

a. Nozzle Size Used: 0.033 inches (inside only).
b. Feed Rate (g/m) for all runs: 22.
c. Head Speed (RPM) for all runs: 1980.
d. Room temperature: 15.6°C (60°F).
e. WR 2721 particle size was reduced to <106 u (utilizing a morter and pestle).
f. WR 2721 dispersed in molten material. Mixture kept under nitrogen blanket.

TABLE 13. MICROCAPSULE PRODUCTION[a,b,c,d,e]

Run No.	Shell Composition [%]	Fill Composition [%]	Feed Rate [g/m] Shell	Feed Rate [g/m] Fill	System [°C]	Theoret. % WR 2721	Analysis % WR 2721	Size [u] Distribution [%] 250-500	500-710	<250+ >710	Comment
1-47B	75.0% Emersol 6349 25.0% Paraffin wax 140/145°F	48.8% Emersol 6349 16.2% Paraffin wax 140/145°F 35.0% WR 2721	11.4	22.0	68.3	23.1	21.1	9.2	58.1	32.7	Made well.
1-47C	62.5% Emersol 6349 37.5% Paraffin wax 140/145°F	40.6% Emersol 6349 24.4% Paraffin wax 140/145°F 35.0% WR 2721	11.4	22.0	68.3	23.1	17.3	11.0	66.7	22.3	Made well.
1-50	70.0% Emersol 6349 30.0% Grocol 600-E	45.4% Emersol 6349 19.6% Grocol 600-E 35.0% WR 2721	11.4	22.0	68.3	23.1	14.9	14.9	69.4	15.7	Made well.
1-51A	30.0% Emersol 6349 70.0% Grocol 600-E	19.6% Emersol 6349 45.4% Grocol 600-E 35.0% WR 2721	11.4	22.0	68.3	23.1	18.0	14.3	44.0	41.7	Made well.
1-51B	50.0% Emersol 6349 50.0% Grocol 600-E	32.5% Emersol 6349 32.5% Grocol 600-E 35.0% WR 2721	11.4	22.0	68.3	23.1	18.6	12.0	65.5	22.5	Made well.
1-52A	50.0% Emersol 6349 50.0% Grocol 600-E	52.0% Emersol 6349 13.0% Paraffin wax 140/145°F 35.0% WR 2721	11.4	22.0	68.3	23.1	19.4	31.9	55.2	31.9	Made well.
1-52B	75.0% Emersol 6349 25.0% Paraffin wax 140/145°F	32.5% Emersol 6349 32.5% Grocol 600-E 35.0% WR 2721	11.4	22.0	68.3	23.1	19.4	12.1	70.2	17.7	Made well.

a. Nozzles Used: 0.033'' inside, 0.046'' outside, 10 mil spacing.
b. WR 2721 particle size was reduced to <106 u (utilizing a mortar and pestle).
c. WR 2721 dispersed in molten fill matrix. Mixture kept under nitrogen blanket.
d. Head Speed (RPM): 1980.
e. Room temperature: 15.6°C (60°F).

TABLE 14. MICROSPHERE AND MICROCAPSULE ANALYSES AND HYDROLYSIS TEST RESULTS (pH 1)

Run No.[a]	Theoretical Payload[b] %	Original Assay[c] %	After Test Assay[d] %	% WR 2721 Recovered[e]	Protection Factor[f] pH 1.0
1-41[g]	35.0	32.7	15.3	46.8	4.6
1-42[g]	35.0	30.7	20.7	67.4	6.7
1-44[g]	40.0	36.1	15.0	41.6	4.1
1-45A[g]	35.0	28.7	8.0	27.9	2.8
1-45B[g]	35.0	25.1	4.0	15.9	1.6
1-45C[g]	35.0	26.3	3.7	14.1	1.4
1-46A[g]	35.0	33.8	4.3	12.7	1.3
1-46B[g]	35.0	34.7	2.4	6.9	0.7
1-46C[g]	35.0	33.4	23.5	70.4	7.0
1-47A[g]	35.0	38.5	13.6	35.3	3.5
1-47B[h]	23.1	21.1	2.3	10.9	1.1
1-47C[h]	23.1	17.3	2.1	12.1	1.2
1-48A[g]	35.0	34.2	3.1	9.1	0.9
1-48B[g]	35.0	32.8	5.6	17.1	1.7
1-49[g]	35.0	33.0	1.0	3.0	0.3
1-50[h]	23.1	14.9	12.3	82.6	8.2
1-51A[h]	23.1	18.0	4.6	25.6	2.5
1-51B[h]	23.1	19.6	9.2	47.0	4.7
1-52A[h]	23.1	19.4	7.3	37.6	3.7
1-52B[h]	23.1	19.4	16.6	85.6	8.5
1-53A[g]	40.0	33.2	6.9	20.8	2.1
1-53B[g]	35.0	32.3	30.6	94.7	9.4
1-54A[g]	35.0	33.4	19.3	57.8	5.7
1-54B[g]	30.0	29.9	16.6	55.5	5.5
1-54C[g]	30.0	28.1	24.4	86.8	8.6
1-55[g]	35.0	32.8	23.9	72.9	7.2

a. Composition given in Tables 1 and 2.
b. Gravimetric composition of melt prior to microsphere or microcapsule production.
c. Determined by HPLC analysis, average of two determinations.
d. Determined by HPLC analysis of recovered microspheres and microcapsules, average of two determinations.
e. Estimated 10.1% of WR 2721 recovered [neat] under similar hydrolysis conditions.
f. Protection factor: % WR 2721 recovered [in microspheres or microcapsules] divided by % WR 2721 recovered [neat] under similar conditions.
g. Microspheres
h. Microcapsules

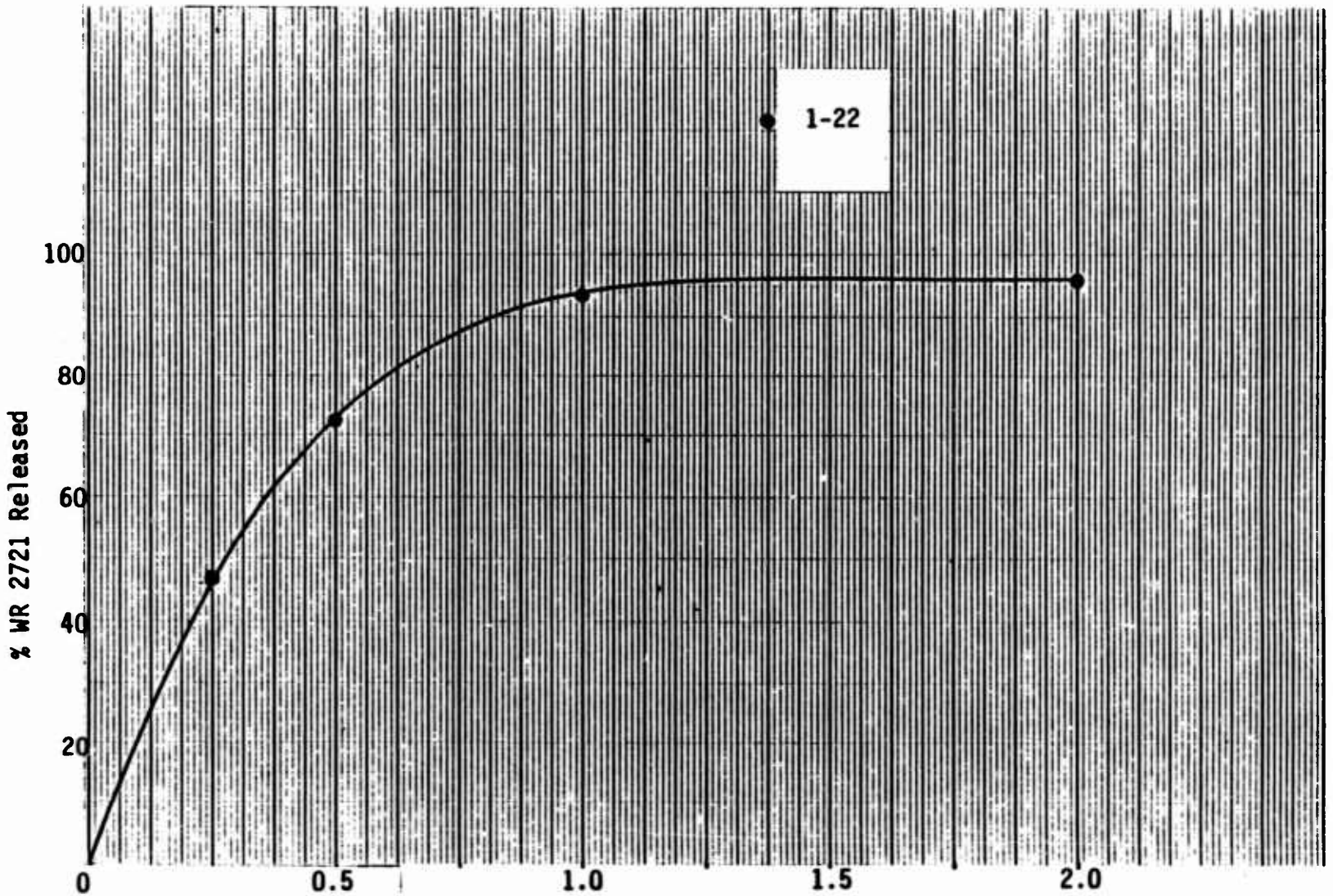


Figure 30. Release of WR 2721 from Microcapsules in Synthetic Intestinal Fluid without Enzymes.

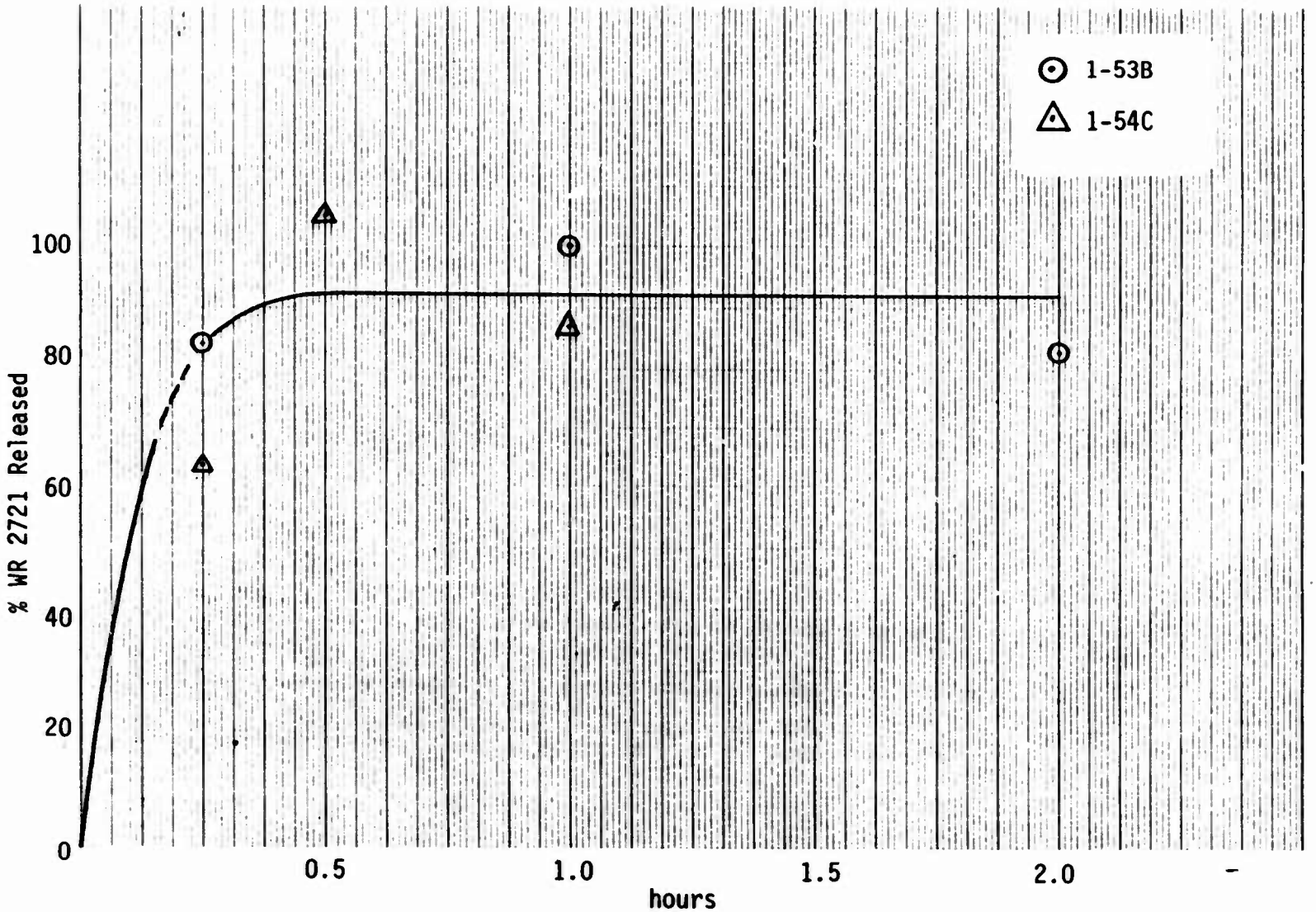


Figure 31. Release of WR 2721 in Microspheres in Synthetic Intestinal Fluid with Enzymes.

TABLE 15. MICROSPHERE AND MICROCAPSULE ANALYSES THERMAL AND HYDROLYSIS TEST RESULTS (pH 1) AFTER WR 2721 CAPSULE SAMPLES HAVE BEEN EXPOSED TO 37°C FOR ONE MONTH

Run No.[a]	Theoretical Payload[b] %	Original Assay[c] %	Assay[d] (%) After 37°C Exposure	% Remaining	Assay[e] (%) After pH 1.0 Test	% Unhydrolyzed[f]	Protection Factor[g] pH 1.0
1-10C[h]	20.0	19.6	15.2	77.6	7.4	48.7	4.8
1-11C[h]	20.0	18.2	14.7	80.8	10.0	68.0	6.7
1-22[i]	14.0	14.1	11.5	81.6	8.6	74.8	7.4
1-27[h]	20.0	19.5	18.4	94.4	4.8	26.1	2.6

a. Detailed composition given in Tables IV and V, Annual Report dated January 1982. (See below also)
b. Gravimetric composition of melt prior to microsphere or microcapsule production.
c. Determined by HPLC analysis, average of two determinations.
d. Determined by HPLC analysis of recovered microspheres and microcapsules, average of two determinations.
e. Determined by HPLC analysis, average of two determinations.
f. Estimated 10.1% of WR 2721 recovered (neat) under similar hydrolysis conditions.
g. Protection factor: % WR 2721 recovered (in microspheres or microcapsules) divided by % WR 2721 recovered (neat) under same conditions.
h. Microspheres.
i. Microcapsules.

Composition

1-10C, 1-27, Microspheres: stearic acid/paraffin matrix
1-11C Microspheres: stearic acid/triglyceride matrix
1-22 Microcapsules: stearic acid/triglyceride matrix

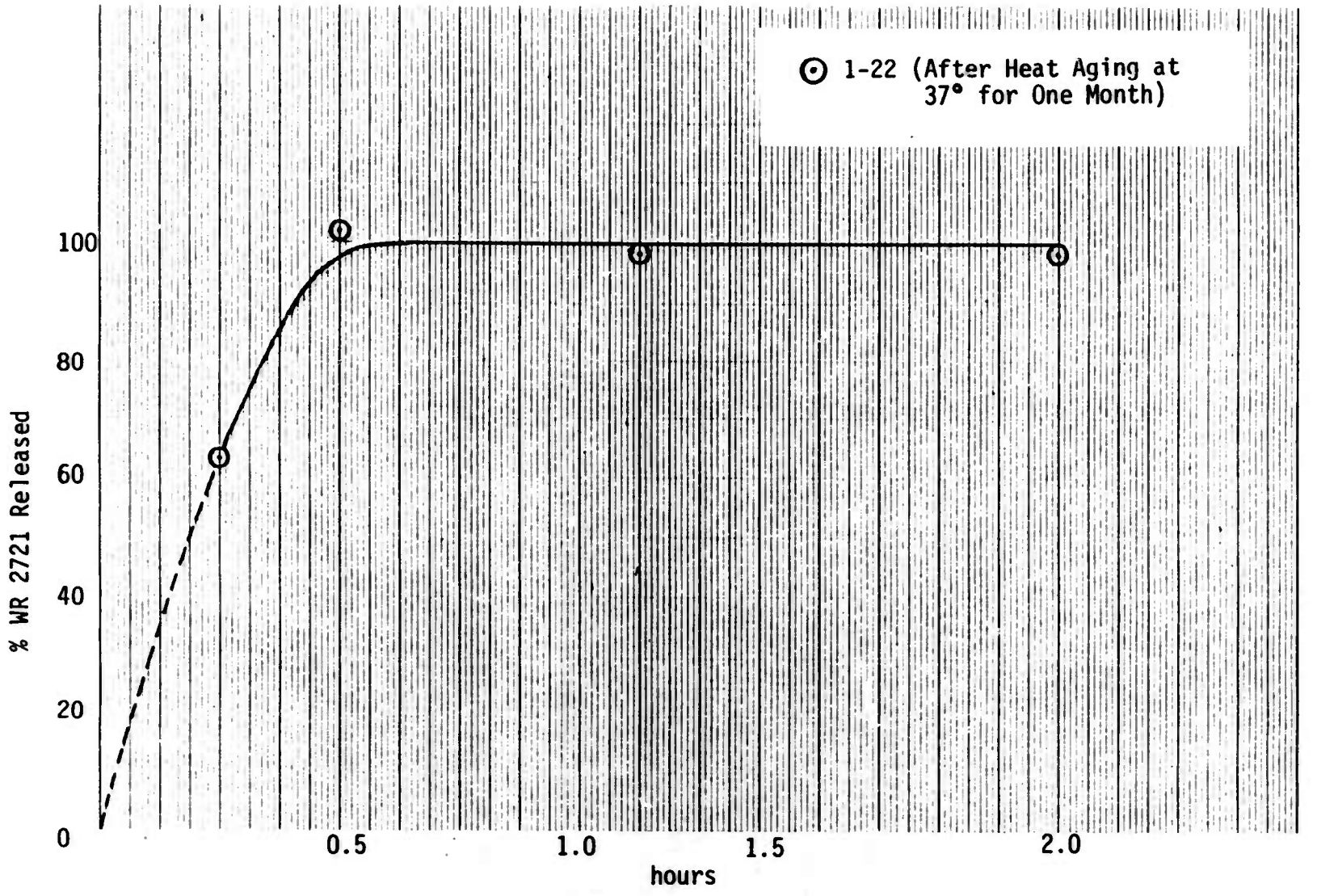


Figure 32. Release of WR 2721 in Microcapsules in Synthetic Intestinal Fluid with Enzyme.

V. DISCUSSION

A. Analytical Methods Development

1. HPLC of WR 2721 and Metabolites

a. PAC Column - Method I

The PAC column used in the assay of WR 2721-containing microspheres could be used to separate the fluorescamine derivatives of WR 2721 and WR 1065 in simple systems. This system was used to study the ability to extract these derivatives from aqueous systems using a variety of organic solvents. It was clear that all systems preferentially extracted WR 1065, leaving the polar WR 2721 fluorescamine derivative in the aqueous layer. It was also evident that WR 2721 was undergoing cleavage to WR 1065, an observation which was expected due to the lability of the drug in water solution.

It is believed that this system is not well suited for the separation of WR 2721 and WR 1065 from the endogenous amines present in plasma. Peak resolution has been poor and column life has been short - even when used in the microsphere assay procedure which involves a much simpler matrix.

b. C-18 Column - Method II

Although WR 2721 and WR 1065, as their fluorescamine derivatives, were separated from each other under the above conditions, they were not separated from interfering substances in canine plasma. The marked chemical dissimilarity of the two compounds did not offer encouragement for the possibility of obtaining a quantitative analysis of each in a single analytical procedure. With sponsor approval, efforts were therefore concentrated upon the development of an analytical procedure for the quantitation of WR 2721 in plasma, with the analysis of RSH (and RSSR) given a lower priority. The following criteria were identified as desirable in the method.

1. Method should be direct, i.e., extraction steps should be avoided.

2. Method should be free of interferences - correction for background should be unnecessary.

3. Analysis time should be short (30 minutes or less). For example, an HPLC method using gradient elution should be avoided.

4. A detection range of 1-1000 μg/mL in plasma was set.

5. The procedure would be designed for quantitation using internal standardization.

To these ends HPLC conditions were found which allowed separation of WR 2721 from interferences in plasma. Under these conditions (described in

Sectopm IV. RESULTS) the RSH derivative was not eluted. The procedure developed does not employ extraction or other sample work-up. This has precedent in the work of DeJong[7], who determined antidepressants in plasma, and Allen, et al.,[8] in their determination of the anthelminthic mebendazole and its metabolites in plasma. Ion-pair chromatography[3], in which an alkylammonium phosphate is added to the HPLC mobile phase, allowed the polar WR 2721 fluorescamine derivative to be retained on the reverse-phase column.

Excellent HPLC separation of WR 2721 from endogenous amines present in dog plasma was obtained using a Waters Associates Radial Compression Module (RCM-100) fitted with a 5 μm C-18 cartridge. This system was characterized by sharp peaks, low back-pressure, high flow rate capability and short equilibration times. Good precision and accuracy was obtained over most of 1-1000 μg/mL concentration range. Only at the low end (<80 μg/mL) of the range was accuracy less than 90% relative to an external standard. This may be due to irreversible binding of a small, constant amount of the drug and not to the analytical procedure. During dosing studies a small interference peak was observed under conditions approaching maximum sensitivity. This peak corresponded to <1 μg/mL of WR 2721 and could be seen as part of a broadened WR 2721 peak in plasma samples containing very low levels of the drug.

This separation system appears to be acceptable for in vivo studies. It requires no sample workup, only a derivatization of the plasma sample, and is accurate and precise over all but lowest extreme of the desired concentration range.

c. C-18 Column - Method III

The fate of WR 2721 in plasma from the time the sample is taken until the drug is detected is unknown. Because of the lability of WR 2721, it cannot be presumed to remain completely intact during this time.

To determine the percentage of WR 2721 remaining at the time of sample analysis relative to that present when the blood sample was obtained an internal standard must be added to the fresh plasma. An internal standard is chosen which shares as many physical and chemical properties as possible with the analyte. Thus, the internal standard will be subject to the same chemical influences as the compound of interest. Occasionally it is not possible to obtain a homologous compound which behaves like the analyte chemically (forms similar derivatives) as well as physically (similar chromatographic behavior, solubility). When this occurs there exists one possible solution - use a suitable isotopically substituted form of the analyte itself. Often this solution may be precluded due to exorbitant cost of preparation of such a compound or nonavailability of the desired isotope. Fortunately, WR 2721 was available which was substituted with ^{14}C, a low energy β emitter.

S-[2-(3-aminopropylamino)ethyl-1,2-^{14}C] phosphorothioic acid (specific activity 86 μCi/mg) was used as the internal standard. At the time of sample collection, a known mass of radioactive WR 2721 was added to the plasma. When the sample was analyzed, the amount of radioactive WR 2721 was again measured. This was achieved by collecting the eluant from the HPLC column and determining the amount of radioactivity associated with the peak corresponding to WR 2721.

The amount of radioactive WR 2721 found remaining at the time of sample analysis divided by the amount added to the plasma at the time of sample preparation represents the fraction of ^{14}C-WR 2721 recovered from the plasma sample. If this fraction is less than 1.00 it represents losses possibly due to chemical reaction, adsorption, partial derivatization and decomposition. As the radiolabeled WR 2721 is for all practical purposes chemically and physically the same as its unlabeled congener, the recoveries obtained for the radiolabeled material may be used to adjust the experimentally determined concentration of the unlabeled WR 2721 to obtain the concentration present in the plasma at the time it was frozen after collection.

d. C-18 Column - Method IV

The discovery of a small interference which almost exactly co-eluted with WR 2721 led to a search for ways to change the relative elution times of WR 2721 and the endogenous amines present in plasma. The search was intensified when it was discovered that hemolysis of the sample added additional components which eluted very near WR 2721, and, depending upon the extent of hemolysis could obliterate the important region of the chromatogram. At constant pH and CH_3CH/water ratio, the nature of the amine used for ion-pairing exerted a large influence on the appearance of the chromatogram. For example, order of elution could be changed by substituting dicyclohexylamine for d-n-hexylamine. Thus far, three ion-pairing reagents have been found which may be used with the RCM-100 CH_3CN/water to separate WR 2721 from all interferences corresponding to greater than about 0.1 µg/mL of the drug, which is the current limit of detectability. Tetrabutylammonium phosphate and di-n-hexylammonium phosphate gave nearly identical chromatograms with the former easier to use in preparation of the mobile phase. WR 2721 elutes between two peaks with near baseline separation when dicyclohexylammonium phosphate is used. All produced chromatograms with open areas which allow for the possibility of an internal standard other than ^{14}C-labeled WR 2721 to be used.

B. In Vivo Studies

The decrease with time in concentration of WR 2721 in plasma is graphically illustrated in Figure 25. This rapid disappearance was well reproduced from experiment to experiment. Inspection of the shape of the decay curves suggested a logarithmic relationship between plasma concentration and post infusion time. The data from both the 10-13-82 and 12-06-82 experiments were analyzed by using the method of least squares to best fit these data to an equation of the form $\ln y_i = b + mx_i$, where y_i is the concentration in µg/mL and x_i is the corresponding post infusion time. The equation and correlation coefficients obtained for each set of experimental data are given below.

Experiment Date	Least Squares Best Fit Equation	Correlation Coefficient (R^2)
10-13-82	ln (concentration) = 6.46-0.056X (Post Infusion Time)	0.94
12-06-82	ln (concentration) = 6.36-0.059X (Post Infusion Time)	0.95

These equations were produced using concentration and time values up to and including the 90 minute post infusion pair. The agreement between the curves obtained from the two sets of experimental data is excellent and lends credence to the repeatability of the analytical method.

C. Microencapsulation

Most of the microencapsulation experiments reported were conducted to determine the maximum amount of WR 2721 that could be incorporated into the microsphere and microcapsule formulations while maintaining desired properties. Maximum payloads were to be between 35 and 40 percent. To achieve these payloads, the as-received WR 2721 had to be milled to reduce in particle size (<105 microns). This classified product provides

- o greater ease of processing
- o improved dispersion
- o improved coating.

Of the various mixtures examined as coatings, matrices containing high percentages of stearic acid afforded the best protection. Microcapsules were prepared in anticipation of obtaining a dosage form with improved physical and chemical properties, especially hydrolytic stability. Satisfactory-appearing microcapsules were obtained in all experiments with a total payload of 23 percent (35 percent in core). Again, the best protection against hydrolysis was obtained with shell systems having a high ratio of stearic acid; however, the degree of protection was about the same as that obtained with microspheres. Further studies are warranted to examine capsule matrices containing higher concentrations (up to 100%) of stearic acid and neat stearic acid as the excipient.

All of the microsphere and microcapsule samples investigated gave complete release of WR 2721 within one or two hours in aqueous solutions at pH 7.4 or in synthetic intestinal fluid. Several of the formulations are very promising for the candidate formulation to be first examined in in vivo oral dosing studies.

Preliminary aging studies indicate that some of the formulations lose small amounts of WR 2721 during aging at 37°C for one month. This may be due to decomposition of the WR 2721 through interaction with the matrix materials; however, further study is required to ascertain the nature of the decomposition.

VI. CONCLUSIONS

1. WR 2721 in plasma can be quantitated over the range of ≤1 to >1000 µg/mL using an HPLC procedure with ^{14}C-labeled WR 2721 as an internal standard. Modification of the procedure so as to use a homologue of WR 2721 as an internal standard appears feasible.

2. WR 2721 appears to have a short initial half-life in the plasma of beagle dogs following IV dosing.

3. WR 2721 can be repeatably encapsulated to protect it against acid hydrolysis and to allow its rapid release at pH 7.5.

VII. RECOMMENDATIONS

1. Continue WR 2721 pilot in vivo IV dosing studies to duplicate results and to confirm accuracy of the assay at low blood plasma levels.

2. Modify the HPLC blood assay method for WR 2721 to use a homologue of WR 2721 as an internal standard.

3. Develop an HPLC blood assay method for WR 1065 and WR 33278.

4. Evaluate the most promising encapsulated capsule samples through bioavailability and pharmacokinetic studies using the beagle dog as animal model.

5. Optimize dosage formulation(s) of WR 2721.

6. Develop sustained-release dosage forms of WR 2721.

7. Continue in vitro evaluation of promising microcapsules by determining stabilities in acid solution and release rates in synthetic intestinal fluids.

8. Continue determination of aging effects at 25 and 37°C on the primary encapsulated samples.

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APPENDIX A

IN VIVO PILOT STUDIES

Mongrel Dog (No. 463) Dosing Study No. 1 October 7, 1982

Protocol Followed dated October 4, 1982

Dog Weight: 40 lbs (18.18 kg)

Dose: 24.5 mL of WR 2721 solution at 125 mg/mL of WR 2721 trihydrate equivalent to 135 mg/kg of WR 2721 (anhydrous)
Total weight dose: 2.45 g anhydrous WR 2721 (3.06 g WR 2721·$3H_2O$)

Dose Infusion Time: 20 seconds, Time started at 10 sec of infusion.

Blood samples: 0 Time (10 mL) and 5 mL at 20, 40 (sec) 1, 2, 3, 6, 9, 15, 30, 45, 60, 90, 120, 150, 180, 210, 240, 300 and 360 min.

NOTES

1. Blood sampling required ~1 minute to withdraw sample. Initial samples were therefore over the following periods.

   20 sec sample: 0-60 sec elapsed time period
   40 sec sample: 60-120
   1 min sample: 120-180
   2 min sample: 180-240
   3 min sample: 240-300

2. Beginning with the ''6 min'' sample and all samples following, the samples were taken at times as indicated.

3. Dog was restrained on table during study and sling was not used.

October 4, 1982

Preliminary Study of Intravenous WR 2721 Pharmacokinetics Using a New HPLC Assay

Background

A new HPLC assay for WR 2721 has been developed for the purpose of conducting bioavailability and pharmacokinetic studies. To demonstrate the suitability of the assay for pharmacokinetic work and to help develop final assay specifications, a preliminary pharmacokinetics study will be undertaken.

Objective

The objective of this pilot experiment is to collect plasma samples to assay for WR 2721 following an intravenous dose of the drug. Plasma samples will be split and assayed on two separate occasions to develop specifications for day-to-day variations in assayed samples.

Materials and Methods

A mature, healthy beagle dog maintained on a regular diet will be used. The diet will be recorded. The dog will be maintained on the diet up to the time of the experiment. Feeding will be once per day at 3-4 p.m. Food will be withheld during the study but the animal will have access to water throughout the experiment (Note 5).

The dog will be restrained on a table throughout the experiment to facilitate drug administration and blood sampling. Tranquilizing drugs will only be used as a last resort if the dog becomes excited.

An indwelling catheter for drug administration will be placed in one of the cephalic veins. Blood samples will be withdrawn from a cannula placed in the opposite jugular vein.

The dose of WR 2721 (150 mg/kg, 187.5 mg/kg trihydrate) will be freshly prepared in normal saline, filtered, with an aliquot saved for drug analysis. The drug will be dissolved in a final volume of 25 mL (conc. = 100 mg/mL; 125 mg/mL trihydrate) and 22.5 mL used as the dosage (Notes 1,4). The drug will be administered over 20 seconds and the catheter will be flushed with 3-5 mL of normal saline.

A blood sample (10 mL) will be collected in EDTA vacutainers before dose injection and blood samples (5 mL) at 0.33 (20 sec), 0.66 (40 sec), 1, 2, 3, 6, 9, 15, 30, 45, 60, 90, 120, 150, 180, 210, 240, 300 and 360 minutes post-injection. The midpoint of injection is set as time zero. The 5-mL blood samples will be collected immediately, centrifuged, divided into two aliquots, spiked with internal standard and stored frozen prior to analysis (Note 6). The blood in the catheters will be replaced with heparinized saline (Note 7). The catheters will be removed at the end of the experiment.

One complete set of each plasma sample will be assayed on two separate occasions to give information on the day-to-day assay varation.

NOTES

1. Dose of 150 mg/kg chosen to stay below toxic effects in dog. Past work at WRAIR has dosed up to 200 mg/kg. Dose for 15 kg dog will be 2.25 g of WR 2721 on a nonhydrated basis (2.81 g as the trihydrate). Total formation prepared will be 3.125 g of WR 2721 as trihydrate dissolved in sufficient physiological saline solution to make 25 mL (125 trihydrate mg/mL).

2. Dose of 150 mg/kg should produce a maximum blood level of WR 2721 of 1500 µg/mL assuming total blood of dog is 1.5 L.

3. For dosing dog, a tranquilizer such as acepromazine or Surital may be used if absolutely necessary.

4. WR 2721 dose will be prepared on date of dosing study. WR 2721 can be weighed out previous to day of study and placed in a rubber septum capped test tube. Physiological saline solution can be added to test tube the day of test and WR 2721 dissolved by agitation. Sample for injection can be removed from tube by syringe fitted with a .45-.50 micron filter (filter to be recommended by L. Fleckenstein).

5. Dog should be fed once/day preferably in afternoon (3-4 p.m.) to better accommodate dosing study. Dose should be on a regular diet.

6. Blood sample immediately after being drawn will be spun down in a refrigerated centrifuge for 10 minutes. The supernatant fluid (plasma) will be removed, divided into two portions and the samples frozen in Dry Ice/isopropyl alcohol (-72°C) and forwarded to Building 70 for storage at -20°C until assayed. Detailed written procedures will be prepared for the assay.

7. The minimum concentration of heparin in saline solution should be used in flushing the catheter. (Recommend 1 cc of 0.0001% heparin solution with 99 cc saline solution).

Beagle Dog (No. 409) Dosing Study No. 2 October 13, 1982

Protocol Followed dated October 11, 1982.

Dog Weight: 31.0 lbs (14.1 kg)

Dose: 21.1 mL of WR 2721 solution at 125 mg/mL of WR 2721 trihydrate equivalent to 150 mg/kg of WR 2721 (anhydrous)
Total dose wt: 2.11 g anhydrous WR 2721 (2.65 g WR 2721·$3H_2O$)

Dose Infusion Time: 2 minutes, Time started after total dose infusion.

Blood samples: 0 Time, (10 mL) and 3 mL at 1, 2, 3, 4, 6, 9, 12, 15, 22, 30, 45, 60, 90, 120, 150, 180, 210, 240, 300 and 360 min.

NOTES

1. Twenty-two minute sample taken from cephalic vein.
2. Vomiting began 15 minutes after dosing and occasionally over a period of thirty minutes.
3. Vomiting after drinking large amount of water around 300 min sampling period.

October 11, 1982

Preliminary Study of Intravenous WR 2721 Pharmacokinetics Using a New HPLC Assay

Background

A new HPLC assay for WR 2721 has been developed for the purpose of conducting bioavailability and pharmacokinetic studies. To demonstrate the suitability of the assay for pharmacokinetic work and to help develop final assay specifications, a preliminary pharmacokinetics study will be undertaken.

Objective

The objective of this pilot experiment is to collect plasma samples to assay for WR 2721 following an intravenous dose of the drug. Plasma samples will be split and assayed on two separate occasions to develop specifications for day-to-day variations in assayed samples.

Materials and Methods

A mature, healthy beagle dog maintained on a regular diet will be used. The diet will be recorded. The dog will be maintained on the diet up to the time of the experiment. Feeding will be once per day at 3-4 p.m. Food will be withheld during the study but the animal will have access to water throughout the experiment (Note 5).

The dog will be restrained on a table throughout the experiment to facilitate drug administration and blood sampling. Tranquilizing drugs will only be used as a last resort if the dog becomes excited.

An indwelling catheter for drug administration will be placed in one of the cephalic veins. Blood samples will be withdrawn from a cannula placed in the opposite jugular vein.

The dose of WR 2721 (150 mg/kg, 187.5 mg/kg trihydrate) will be freshly prepared in normal saline, filtered, with an aliquot saved for drug analysis. The drug will be dissolved in a final volume of 25 mL (conc. = 100 mg/mL; 125 mg/mL trihydrate) and 22.5 mL used as the dosage (Notes 1,4). The drug will be administered over 20 seconds and the catheter will be flushed with 3-5 mL of normal saline.

A blood sample (10 mL) will be collected in EDTA vacutainers before dose injection and blood samples (3 mL) at 1, 2, 3, 6, 9, 12, 15, 22, 30, 45, 60, 90, 120, 150, 180, 210, 240, 300 and 360 minutes-post injection. The end of the infusion period (2 min) is set as time zero. The blood samples will be collected immediately cooled in ice water, centrifuged, divided into two aliquots, spiked with internal standard and stored frozen prior to analysis (Note 6). The blood in the catheters will be replaced with heparinized saline (Note 7). The catheters will be removed at the end of the experiment.

One complete set of each plasma samples will be assayed on two separate occasions to give information on the day-to-day assay variation.

NOTES

1. Dose of 150 mg/kg chosen to stay below toxic effects in dog. Past work at WRAIR has dosed up to 200 mg/kg. Dose for 15 kg dog will be 2.25 g of WR 2721 on a nonhydrated basis (2.81 g as the trihydrate). Total formation prepared will be 3.125 g of WR 2721 as trihydrate dissolved in sufficient physiological saline solution to make 25 mL (125 trihydrate mg/mL).

2. Dose of 150 mg/kg should produce a maximum blood level of WR 2721 of ~1500 μg/mL assuming total blood of dog is 1.5 L.

3. For dosing dog, a tranquilizer such as acepromazine or Surital may be used if absolutely necessary.

4. WR 2721 dose will be prepared on date of dosing study. WR 2721 can be weighed out previous to day of study and placed in a rubber septum capped test tube. Physiological saline solution can be added to test tube the day of test and WR 2721 dissolved by agitation. Sample for injection can be removed from tube by syringe fitted with a .45-.50 micron filter (filter to be recommended by L. Fleckenstein).

5. Dog should be fed once/day preferably in afternoon (3-4 p.m.) to better accommodate dosing study. Dose should be on a regular diet.

6. Blood sample immediately after being drawn will be cooled in ice water and spun down in a refrigerated centrifuge for 10 minutes. The supernatant fluid (plasma) will be removed, divided into two portions and the samples frozen in Dry Ice/isopropyl alcohol (-72°C) and forwarded to Building 70 for storage at -20°C until assayed. Detailed written procedures will be prepared for the assay.

7. The minimum concentration of heparin in saline solution should be used in flushing the catheter. (Recommend 1 cc of 0.0001% heparin solution with 99 cc saline solution).

Beagle Dog (No. 409) Dosing Study No. 3 December 6, 1982

Protocol Followed dated December 6, 1982

Dog Weight: 28.0 lbs (12.72 kg)

Dose: 15.9 mL of WR 2721 solution at 150 mg/mL[a] of WR 2721 trihydrate equivalent to 150 mg/kg of WR 2721 (anhydrous)
Total dose weight: 1.908 g (anhydrous WR 2721) 2.385 g WR 2721·$3H_2O$

Blood samples: 0 Time, (10 mL taken 23 minutes before dose infusion) and 1, 2, 3, 6, 9*, 12, 15, 22, 30*, 32**, 45, 60, 90, 120, 150, 180, 210, 240, 300, and 360 min (post injection).

* Small due to sampling problems
**Extra sample

a. Should have been at 125 mg/mL concentration - error in formulation.

December 6, 1982

Preliminary Study of Intravenous WR 2721 Pharmacokinetics Using a New HPLC Assay

Background

A new HPLC assay for WR 2721 has been developed for the purpose of conducting bioavailability and pharmacokinetic studies. To demonstrate the suitability of the assay for pharmacokinetic work and to help develop final assay specifications, a preliminary pharmacokinetics study will be undertaken.

Objective

The objective of this pilot experiment is to collect plasma samples to assay for WR 2721 following an intravenous dose of the drug. Plasma samples will be split and assayed on two separate occasions to develop specifications for day-to-day variations in assayed samples.

Materials and Methods

A mature, healthy beagle dog maintained on a regular diet will be used. The diet will be recorded. The dog will be maintained on the diet up to the time of the experiment. Feeding will be once per day at 3-4 p.m. Food will be withheld during the study but the animal will have access to water throughout the experiment (Note 5).

The dog will be restrained on a table throughout the experiment to facilitate drug administration and blood sampling. Tranquilizing drugs will only be used as a last resort if the dog becomes excited.

An indwelling catheter for drug administration will be placed in one of the cephalic veins. Blood samples will be withdrawn from a cannula placed in the opposite jugular vein.

The dose of WR 2721 (150 mg/kg, 187.5 mg/kg trihydrate) will be freshly prepared in normal saline with an aliquot saved for drug analysis. The drug will be dissolved in a final concentration of 100 mg/mL WR 2721 or 125 mg/mL WR 2721·$3H_2O$. The drug will be administered over two (2) minutes and the catheter will be flushed with 3-5 mL of normal saline.

A blood sample (10 mL) will be collected in EDTA vacutainers before dose injection and blood samples (3 mL) at 1, 2, 3, 6, 9, 15, 30, 45, 60, 90, 120, 150, 180, 210, 240, 300 and 360 minutes post injection. The end of the infusion period (2 min) is set as time zero. The blood samples will be collected immediately, cooled in ice water, centrifuged, divided into two aliquots, spiked with internal standard and stored frozen prior to analysis (Note 6). The blood in the catheters will be replaced with heparinized saline (Note 7). The catheters will be removed at the end of the experiment.

One complete set of each plasma samples will be assayed on two separate occasions to give information on the day-to-day assay varation.

NOTES

1. Dose of 150 mg/kg chosen to stay below toxic effects in dog. Past work at WRAIR has dosed up to 200 mg/kg. Dose for 15 kg dog will be 2.25 g of WR 2721 on a nonhydrated basis (2.81 g as the trihydrate). Total formation prepared will be 3.750 g of WR 2721 as trihydrate dissolved in sufficient physiological saline solution to make 30 mL (125 trihydrate mg/mL). Dose would be 22.5 mL).

2. Dose of 150 mg/kg should produce a maximum blood level of WR 2721 of ~1500 µg/mL assuming total blood of dog is 1.5 L.

3. For dosing dog, a tranquilizer such as acepromazine or Surital may be used if absolutely necessary.

4. WR 2721 dose will be prepared on date of dosing study. WR 2721 can be weighed out previous to day of study and placed in a rubber septum capped test tube. Physiological saline solution can be added to test tube the day of test and WR 2721 dissolved by agitation. Sample for injection can be removed from tube by syringe.

5. Dog should be fed once/day preferably in afternoon (3-4 p.m.) to better accommodate dosing study. Dose should be on a regular diet.

6. Blood sample immediately after being drawn will be cooled in ice water and spun down in a refrigerated centrifuge for 10 minutes. The supernatant fluid (plasma) will be removed, divided into two portions and the samples frozen in Dry Ice/isopropyl alcohol (-72°C) and forwarded to Building 70 for storage at -20°C until assayed. Detailed written procedures will be prepared for the assay.

7. The minimum concentration of heparin in saline solution should be used in flushing the catheter. (Recommend 1 cc of 0.0001% heparin solution with 99 cc saline solution).

APPENDIX B

MATERIALS LIST

Description	Source	Lot Number
S-2-(3-Aminopropylamino)-ethyl-phosphorothioic acid [WR 2721]	Walter Reed Army Institute Research (WRAIR)	AX BK 02762 PB-V-116
Emersol 6349 [stearic acid food (70) good grade]	Emery Industries, Inc.	21375 and 1606
Grocol 600-E [hydrogenated tallow glycerides food grade]	A. Gross and Co.	6-21459 and B.G. 1075-E
Softisan 154 [hydrogenated tallow glycerides good grade]	Kay-Fries, Inc. (Dynamit Nobel)	161816
Atmul 84K [mono- and diglycerides of edible oils and fats]	ICI United States, Inc. (Kraft Industrial Foods Division)	8103

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SUPPLEMENTARY

INFORMATION

DEPARTMENT OF THE ARMY
U.S. ARMY MEDICAL RESEARCH AND DEVELOPMENT COMMAND
FORT DETRICK, FREDERICK, MD 21702-5012

ERRATA

REPLY TO
ATTENTION OF:

SGRD-RMI-S (70-1y) AD B094 912

31 JUL 1992

MEMORANDUM FOR Administrator, Defense Technical Information Center, ATTN: DTIC-HDS/William Bush, Cameron Station, Bldg. 5, Alexandria, VA 22304-6145

SUBJECT: Request Change in Distribution Statement

1. The U.S. Army Medical Research and Development Command (USAMRDC), has reexamined the need for the limited distribution statement on technical reports for Contract No. DAMD17-80-C-0128. Request the limited distribution statement for AD Nos. ADB063672, ADB080528, ADB094912, ADB094846, ADB109071, and ADB109038, be changed to "Approved for public release; distribution unlimited," and that copies of these reports be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Virginia Miller, DSN 343-7325.

ERRATA

CAREY O. LEVERETT
LTC, MS
Deputy Chief of Staff for
Information Management

CF:
Dr. Ruthann Smejkal/WRAIR

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